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CONTENTS

No. 1 JULY, 1937

No. 2 AUGUST, 1937

No. 3 SEPTEMBER, 1937

No. 4 OCTOBER, 1937

No. 5 NOVEMBER, 1937

No. 6 DECEMBER, 1937

THE EFFECT OF THE ADMINISTRATION OF ACID AND ALKALINE SALTS UPON THE ASCORBIC ACID CONTENT OF GUINEA PIG TISSUES

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In a previous study of the urinary excretion of ascorbic acid (vitamin C) by normal human subjects, it was observed that the administration of acid and alkaline salts was followed by striking variations in the amount of ascorbic acid found in the urine (Hawley, Frazer, Button and Stephens, '36). In subjects who had been previously saturated with vitamin C, the administration of sodium bicarbonate was followed by the excretion of approximately 50% of daily test doses of 100 mg. of ascorbic acid. The substitution of ammonium chloride for sodium bicarbonate resulted in the excretion of a highly acid urine and increase in the daily excretion to approximately 100% of the daily dose of ascorbic acid.

In a preliminary experiment seventy guinea pigs, part of which had been maintained for several weeks on a diet partially deficient in vitamin C during the course of another study, were divided into groups and were given the crystalline ascorbic acid, the ascorbic acid together with $NaHCO_3$ or NH_4Cl or the salts alone, as indicated in table 1.

In all experiments the animals were sacrificed exactly 2 hours after the administration of the ascorbic acid which was given exactly 1 hour after the $NaHCO_3$ or NH_4Cl. The animals were killed and the livers analyzed for ascorbic acid according to the method outlined below. The average amount

of ascorbic acid found in the liver in each group is shown in table 1.

These observations suggested that retention of ascorbic acid in the liver was increased by the administration of the alkaline salt. The following experiment was designed to further investigate the possible relation of the administration of acid and alkaline salts to the amount of ascorbic acid retained in the liver and adrenals of the guinea pig.

TABLE 1

Ascorbic acid in milligrams per 100 gm. liver

'Positive controls' of original experiment—normal animals

Control group	11.25
Ammonium chloride	11.08
Ammonium chloride + 10 mg. ascorbic acid	11.20
Sodium bicarbonate	11.15
Sodium bicarbonate + 10 mg. ascorbic acid	17.55

Animals on partially deficient diets

$NaHCO_3$ GROUP		$NHCl_4$ GROUPS	
Negative controls	6.63	....	
5 mg. ascorbic acid [1]	7.28	5 mg. ascorbic acid	7.68
$NaHCO_3$ + 5 mg. ascorbic acid	9.18	NH_4Cl + 5 mg. ascorbic acid	8.42
Negative controls	6.60	Negative controls	6.08
10 mg. ascorbic acid	9.57	10 mg. ascorbic acid	8.02
$NaHCO_3$ + 10 mg. ascorbic acid	11.80	NH_4Cl + 10 mg. ascorbic acid	7.53

[1] In this series the crystalline ascorbic acid was administered rather than the salt.

METHOD

Guinea pigs weighing from 200 to 300 gm. were placed on the Sherman vitamin C deficient diet [1] for a period of 17 days. This was considered a sufficient period of time to deplete the vitamin C stores and induce mild scurvy without resulting in marked pathological changes. At the end of the depletion

[1] Wheat bran 29.5 parts; ground rolled oats 29.5 parts; skim milk powder (heated in shallow trays for 5 hours at 110°C.) 30 parts; sodium chloride 1.0 part; butter fat 8.0 parts; cod liver oil 2.0 parts.

period the animals were divided into six groups as indicated below:

Group A, killed at the end of the 17-day depletion period.

Group B, fed 10 mg. of ascorbic acid daily. Killed in sub-groups after 2, 4, 6, 15 and 23 days.

Group C, fed 10 mg. of ascorbic acid and 32.5 mg. of ammonium chloride daily. Killed in sub-groups after 2, 4, 6 and 15 days.

Group D, fed 10 mg. of ascorbic acid and 100 mg. of sodium bicarbonate daily. Killed in sub-groups after 2-, 4-, 6-, 8-, and 15-day periods.

Group E, adequate intake of C for 40 days (17 + 23 days).

The sixth group of guinea pigs, group F, was fed without previous depletion a diet adequate in vitamin C, with an added daily ration of 5 cc. of orange juice for a period of 45 days, at the end of which time they too were sacrificed.

At the end of each experimental period the animals were chloroformed, autopsied and examined according to the method of Sherman, LaMer and Campbell ('22). The degree of scurvy in each animal was expressed numerically as the sum of the plus signs recorded in the scoring. The tissues were quickly removed, weighed, ground with fine sand and extracted by vigorous shaking for 5 minutes with a 20% acid mixture (16% trichloracetic acid, 4% metaphosphoric acid). Sufficient water was then added to reduce the acidity to 5% and the acid tissue mixture was shaken for 10 additional minutes, after which it was filtered. The filtrate was titrated against a standardized solution of 2, 6-dichlorobenzenoneindophenol.[2] The results were expressed as milligram per cent ascorbic acid. The hydrogen ion concentration was determined by the colorimetric method in urine aspirated from the bladder at the time of autopsy.

RESULTS

Marked individual variations in the ascorbic acid content of guinea pig tissues were observed. It is sometimes assumed that results obtained by the chemical method in controlled groups of animals are more or less uniform. Table 2 is inserted to illustrate the wide variations which are found

[2] Practical substitute for 2, 6-dichlorphenolindophenol, Eastman Kodak Co.

TABLE 2

Ascorbic acid content of guinea pig tissues reported in the literature

INVESTIGATORS	DIET	NUMBER OF ANIMALS	MILLIGRAMS ASCORBIC ACID PER 100 GM.	
			Adrenal	Liver
Diets adequate in vitamin C				
Bessey and King ('33)	C free + excess spinach for 10 days	?	75	10
Yavorsky, Almaden and King ('34)	C free + excess spinach for 10 days	8	70	10
Birch and Dann ('33)	Normal diet	?		30
Svirbely ('33)	C free + spinach for 21 days	4	124 (114–135)	23 (11–32)
Fox and Levy ('36)	Cabbage and bran	10	150 (110–206)	25
Fox and Levy ('36)	Same + 100 mg. ascorbic acid for 8 days	2	201 (196–206)	40 (38–42)
Jacobsen ('35)	C free diet + 20 mg. ascorbic acid for 9 days	2	38	7.7
Jacobsen ('35)	Same for 3 months	2	18	6.2
Jacobsen ('35)	Same + cabbage ad lib for 3 months	2	78	25
Hawley, Daggs and Stephens	Adequate diet + 5 cc. orange juice for 45 days	5	39 (22–77)	25 (11–28)
Diets inadequate in vitamin C				
Bessey and King ('33)	C free for 15 days	?	10	4.3
Yavorsky, Almaden and King ('34)	C free for 15 days	6	8	3
Yavorsky, Almaden and King ('34)	C free for 28 days	6	3	1
Birch and Dann ('33)	'Scorbutic'	?		9
Svirbely ('33)	C free for 21 days	4	7 (6–10)	2.0
Hawley, Daggs and Stephens	C free for 17 days	10	12.6 (9–16)	2.7 (2–4)

in the literature. Table 3 shows the variations encountered in the individual animals within a single group, the 'F' group. These animals had received a diet of alfalfa hay, whole oats and cabbage. To this was added a daily ration of 5 cc. of orange juice in an attempt to completely saturate the tissues with vitamin C. The saturation point for individual animals evidently varied within wide limits. The ascorbic acid content of the tissues of this group of animals showed greater variations than any of the other groups. This, we feel, emphasizes the value of preliminary standardization by depletion in a study such as this before the experimental diet is fed.

The results summarized in tables 4 and 5 appear to confirm the observations of the preliminary experiment. They indicate that the administration of sodium bicarbonate with

TABLE 3

Variations in ascorbic acid content of organs from animals within the same test group (F)

Animal no.	1	2	3	4	5	AVERAGE
						mg.%
Liver	27.9	16.0	14.2	11.3	10.0	16.02
Adrenal	21.5	76.5	41.0	32.1	25.9	39.40

ascorbic acid increases the retention of the vitamin. They further suggest that when the vitamin is ingested in its natural form as cabbage, alfalfa hay and orange juice (F group) that there is even better vitamin retention by the tissues. Jacobsen ('35) likewise found in his series that the tissue content of those animals receiving the basal diet plus cabbage ad lib (equivalent to approximately 20 mg. ascorbic acid daily) was definitely higher in ascorbic acid than was the tissue of the animals receiving the equivalent amount of ascorbic acid in its crystalline form.

After the sixth day, the adrenals and, to a lesser extent, the livers of those animals which had received sodium bicarbonate contained significantly larger amounts of ascorbic acid than those animals which had received ammonium chloride or those which were given ascorbic acid alone. Furthermore, the

TABLE 4

Summary of present experimental data

GROUP	CONDITION	TEST DAYS	NUMBER OF ANIMALS	MILLIGRAMS PER CENT ASCORBIC ACID In liver	MILLIGRAMS PER CENT ASCORBIC ACID In adrenals	COMPOSITE URINARY pH	AVERAGE SCURVY SCORE	WEIGHT CHANGE IN GRAMS
A	C free diet	17	10	2.65 (1.58–4.03)	12.46 (9.30–15.50)		9	— 6
B	Ascorbic acid added	2	9	6.92 (5.13–8.43)	14.13 (11.45–17.20)		12	— 33
		4	9	6.94 (4.43–10.90)	17.83 (9.10–25.00)	6.9	10	— 25
		6	9	7.84 (5:35–10.15)	21.36 (14.95–33.20)		7	— 10
		15	7	8.01 (7.00–9.40)	16.88 (9.30–24.10)	6.8	1—	+ 21
C	Ascorbic acid + NH_4Cl	2	8	7.79 (7.10–9.75)	13.92 (12.05–17.10)	6.0	13	— 24
		4	9	5.85 (5.00–7.50)	16.92 (12.00–21.40)	6.1	9	— 27
		6	8	6.87 (4.60–9.75)	21.34 (11.55–36.20)	6.4	11	— 21
		15	8	7.36 (4.80–10.00)	15.04 (8.60–33.40)	5.6	1—	+ 68
D	Ascorbic acid + $NaHCO_3$	2	3	7.10 (5.85–8.80)	15.27 (13.90–17.70)	7.4	18	— 36
		4	6	4.81 (3.25–6.35)	16.68 (14.00–20.90)	8.0	8	— 4
		6	5	8.64 (5.05–13.25)	34.12 (19.90–46.80)		3	— 14
		8	5	10.42 (6.92–15.86)	25.32 (18.40–33.80)	7.8	1	+ 21
		15	7	10.03 (7.00–12.60)	32.07 (26.20–40.60)	8.0	1—	+ 48
E	Adequate diet	40	5	10.15 (5.50–17.70)	20.71 (10.50–35.00)	7.6	0	+ 100
F	Adequate C + orange juice	45	5	16.02 (10.70–27.90)	39.40 (21.50–76.50)	8.0	0	+ 150

scurvy scores suggest that the additional ascorbic acid retained by those animals which received the alkaline salt was utilized to prevent or correct manifestations of scurvy. The changes in the urinary hydrogen ion concentration were comparable to those observed in human subjects (Hawley, Frazer, Button and Stephens, '36).

TABLE 5

Summary of average values for various organs in the A, E and F groups

GROUP	LIVER	ADRENAL	KIDNEY	HEART	SPLEEN	BRAIN	EYES	OVARIES	TESTES	BLADDER	SCURVY SCORE	NUMBER ANIMALS	URINE pH
A[1]	2.65	12.46	1.92	2.86	5.97	3.99	2.59		3.91	10.62	9	10	?
E[2]	10.15	20.71	5.04	3.54	7.21	11.94	3.94	17.00	16.30	10.25	0	5	7.6
F[3]	16.02	39.40	7.90	4.70	18.00	7.48					0	5	8.0

[1] C free diet.

[2] Adequate diet.

[3] Adequate diet plus orange juice.

SUMMARY

In a previous study the authors observed a definite relationship, in humans, between the amount of ascorbic acid found in the urine and the hydrogen ion concentration of the latter. Observations in guinea pigs show that the administration of sodium bicarbonate in amounts sufficient to result in a highly alkaline urine results in an increased concentration of ascorbic acid in the adrenals and liver. It is shown that increased retention of ascorbic acid in the tissues may be at least a partial explanation of the decreased urinary output of ascorbic acid observed in humans in whom the urine had been rendered alkaline by the administration of sodium bicarbonate.

The authors wish to take this opportunity to express their appreciation to Merck & Company who generously supplied the ascorbic acid used in this experiment.

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COMPARATIVE EXPERIMENTS WITH CANNED, HOME COOKED, AND RAW FOOD DIETS [1]

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Much has been said and written about the relative merits of raw and cooked foods. Probably the difficulty of securing experimental evidence on such a question is the reason for the limited concrete data. Three comprehensive series of animal experiments are herewith presented. The first was conducted over a number of successive generations. To eliminate a possible hereditary effect a second plan limited to one generation was followed. That any variable due to variation in lacteal performance might be eliminated, a third was limited to the period of rapid growth after weaning.

Vitamins bring cooking into question

With the discovery of the vitamins, there came a questioning of the cooking of foods. The canning of foods generally requires, for purposes of sterilization, more thorough cooking than is otherwise practiced. Studies on the effect of the canning process on the various vitamins in our most common vegetables and fruits have shown that cooking need not be deleterious to vitamins. In fact, insofar as canning serves to promote a greater consumption of vegetables and fruits, it tends to enhance our vitamin intake. Tressler, Mack and King ('36) recently conclude that in cooking "the total amount

[1] Presented before the Division of Biological Chemistry, American Chemical Society, Chapel Hill, North Carolina, April, 1937.

of ascorbic acid decreases but little,'' calling attention at the same time to the fact that ''a considerable proportion of the ascorbic acid passes into the water during the boiling of vegetables.'' In the experiments here recorded the cooking water was held to a minimum and fed with the food cooked in it.

Initial experiment with all canned food diet

A study of the vitamins cannot, however, give a comprehensive answer to the question of the effect of cooking foods, since the necessary dietary constituents comprise a great many other factors—some undoubtedly as yet not recognized. Physical factors such as texture, consistency, colloidal conditions, etc., and possibly the activity of enzymes are also involved. It was therefore decided to feed two species of animals—guinea pigs and rats—a diet made up wholly of canned foods through a number of generations. While it may be argued that man, through his experience over many centuries, has undergone physiological changes to adapt himself to cooked foods, such a statement would not be pertinent to these two species of animals. If canning adversely affects the nutritive value of foods, it would be expected to show up after a few generations. No such evidence developed. As a matter of fact, the guinea pigs through eight generations, and the rats through ten generations, thrived better than usual. Growth rates and weights were above those recorded as average (Kohman, Eddy and Gurin, '31).

Plan for feeding canned, home cooked, and raw foods comparatively

The outcome of this experiment suggested the desirability of conducting more comprehensive experiments in which canned foods, ordinary (home) cooked foods, and raw foods were to be fed on a comparable basis. Rats have been used in such experiments. They were planned to conform as nearly as possible to the consumption of foods under usual conditions. The junior author (M.W.) in charge of the animals

was instructed to purchase all foods off the New York market just as they would be purchased for household use, and cook those for the home cooked diet according to directions obtained from the Foods and Cookery Department of Teachers College. The same group of foods was always purchased for each of the three diets. It should be borne in mind that the raw and home cooked diets represented the same raw products since purchases off the New York market were simply divided to constitute the two diets. The canned food diet represents different lots of the same food and there was no record of where or when they were canned except that they represented canned foods on the market at the time. Each group of foods used comprised different types of food to provide what is considered a balanced meal. To simplify matters, a given group of foods was usually employed for a period of a few days to a week. Milk was not used because of its ability to cover up dietary deficiencies. Food mixtures and proprietary products, the components of which were uncertain, were avoided. Typical groups of combinations of foods used are:

Roast beef, white potatoes, green beans, tomatoes, peaches
Tongue, sweet potatoes, peas, pumpkin, blueberries
Chicken, noodles, okra, turnip greens, strawberries

In about fifty similar combinations, practically all the common vegetables, fruits, meat and fish products were used. All the experiments conducted on this general subject in different ways have covered a period of over 4 years and therefore took into account any seasonal variation.

These combinations were fed to the experimental animals by putting each food in a separate dish and allowing each animal a free choice. Grinding or macerating the food for mixing into an homogeneous mixture did not seem warrantable because in the case of raw foods rapid enzymic changes would be induced. Moreover, maceration is part of the digestive process. There is considerable evidence that animals have the ability to choose diets to accomplish optimum results if suitable foods are available. Davis ('28) obtained

excellent results by allowing infants a free choice of their foods. Franke and Potter ('36) found that rats always chose a normal wheat in preference to one grown on seleniferous soil and containing selenium to the extent of 30 parts per million. In case of mixtures of the two the mixtures containing the least selenium-bearing wheat were chosen.

The evidence of five generations

Our first plan of experimentation was to start three pairs of rats on each diet, using litters of weaning age and distributing them between the diets as equally as possible both as to

TABLE 1

Reproductive record of rats on raw, home cooked and canned food

	FOOD		
	Raw	Home cooked	Canned
Total number of litters[1]	67	51	60
Litters dead before weaning, per cent	12	6	5
Number of young at birth	476	405	482
Young dead before weaning, per cent	22.2	12.4	12.00
Average number per litter at birth	7.10	7.94	8.00
Average weight of young at birth, grams	4.68	4.96	5.28
Average weight of young when weaned, grams	20.5	23.0	27.3
Average weight when 60 days old	73	71	89
Average weight when 90 days old	125	125	149

[1] The home cooked group were started somewhat later than the other two and hence not with litter mates of the other two but of the same stock. Hence total numbers cannot be compared with the other two groups.

weight and as to sex. It should be understood, however, that the group on home cooked foods was started somewhat later than the other two as an after thought. Total numbers are therefore not to be compared with similar numbers of the other two groups. This group, while not started with litter mates of the other two, did originate from the same stock. Through five generations close to 500 animals were produced on each diet. The records of this experiment are given in table 1 which is abbreviated from the original report (Kohman, Eddy and associates, '34).

It was particularly interesting that a determination of the percentage of bone ash of the alcohol-ether extracted dried tibia of a considerable number of animals disclosed a distinctly higher degree of calcification resulting from a canned food diet. This point was therefore made the subject of the experiments recorded below.

Single generation experiments to eliminate influence of heredity

One weakness in the above line of experimentation is that all the animals on each diet originated from three pairs. Even though these pairs represented litter mates, as was the case of animals on canned and raw foods, respectively, it might still be argued that heredity might have played a considerable role. To meet this weakness, an entirely different procedure was employed. Litter mates were again used to represent the different diets. Mature animals were bred so as to obtain at least three litters on the same day. The young of each of the three litters were then equally divided between the three mothers, and one mother with its young placed on raw foods, another mother with its young on a portion of these same foods after kitchen cooking and the third mother with its young on a canned food diet. When the young were weaned at 21 days of age, they were kept on their respective diets until 60 days of age for both growth and bone ash records. In a subsequent experiment, the particular mother that had reared her young on a raw food diet, for example, was placed on one of the other two diets and in still another experiment, on the third diet, so as to bring about uniformity of animals on the three diets as far as possible. In experiment 5, table 2, in addition to dividing the young of each litter on the day of birth between the three mothers, at the end of the first week the mothers but not the young were shifted to one of the other two diets and at the end of the second week to the third diet. Fortunately, the maternal characteristics of the animals permitted this without any disturbance. The various experi-

ments conducted with this procedure are summarized in table 2.

The outstanding fact from these data is the distinctly better or higher percentage of bone ash resulting from the canned

TABLE 2

Average bone ash of the alcohol ether extracted dried tibia of 60-day-old animals on canned, home cooked and raw food diets, respectively

EXPERIMENT NO.	DATE OF EXPERIMENT AND DIET	NUMBER OF ANIMALS	AVERAGE WEIGHT OF ANIMALS	BONE ASH OF TIBIA	PROBABILITY OF NON-SIGIFICANCE OF DIFFERENCE IN BONE ASH BETWEEN ANIMALS ON CANNED FOODS AND	
					Home cooked foods	Raw foods
1	July 7, 1934		*gm.*	%		
	Canned	9	110	42.0	< 0.01	< 0.01
	Home cooked	9	106	36.6		
	Raw	9	101	36.9		
2	July 10, 1934					
	Canned	8[1]	76	37.9	0.10	0.74
	Home cooked	9	81	35.4		
	Raw	9	89	37.5		
3	Oct. 15 1934					
	Canned	6	168	51.2	0.15	< 0.01
	Home cooked	4[1]	199	50.1		
	Raw	5[1]	174	45.4		
4	Oct. 16, 1934					
	Canned	9	112	49.6	< 0.01	< 0.01
	Home cooked	9	98	43.8		
	Raw	9	92	40.9		
5	March 4, 1935					
	Canned	6	140	48.4	< 0.01	< 0.01
	Home cooked	6	154	45.3		
	Raw	6	131	43.1		
6	March 4, 1935					
	Canned	8	153	51.0	< 0.01	< 0.01
	Home cooked	8	130	44.4		
	Raw	8	126	47.0		

[1] The reason for unequal numbers of animals in this experiment is due to deaths before weaning time on these diets.

food diet, comparable to what was manifest in the previous line of experimentation. There is apparent a fluctuation in the weight attained by the animals on the three respective diets which may be no more than the normal variations to be

expected. At any rate, there is no consistent difference in weights attained on the three diets.

The weight up to this age (60 days) is not independent of the nursing period during which only the mothers eat of the respective diets. There is no means of knowing how much variation in lacteal performance of the mother might have affected the results. Whatever the weight attained, there is a striking consistency in the higher percentage of bone ash in the animals on the canned food diet. Another weakness in this plan of experimentation is that although the three mothers were given an equal number of young in the beginning of each experiment, an occasional death occurred before the weaning period. Since during this period the young animals depend almost exclusively on the milk of their mother, this reduction of the number of the litter gave the remaining animals an advantage. This is clearly reflected in the weight of the animals in experiment 3 in table 2. As is apparent from the data, no deaths occurred from the litter on the canned food diet which might have worked to the advantage of the remaining animals since that would leave a smaller number to subsist on the milk of the mother. In spite of this, the average percentage of bone ash is distinctly higher for those animals reared on canned foods. The greater weight attained on each diet in this experiment than in the others no doubt reflects lacteal advantage of small litters.

Experiments covering rapid growth period after weaning

To meet the weakness of this line of experimentation, another series of experiments was conducted on still another plan. Stock animals were taken from the Teachers College, Columbia University colony at a rather early weaning period of 21 days of age. Litter mates were again divided for uniformity both as to sex and weight between the three diets to secure six animals on each diet for each of six experiments covering a period of about a year. These were all fed to an age of 60 days, when the growth and bone ash records were obtained. The data are summarized in table 3.

While there is some fluctuation between the raw and home cooked food animals, again in each experiment there is a consistently higher percentage of bone ash in those animals receiving the canned food diet.

TABLE 3

Average bone ash of the alcohol ether extracted and dried tibia of six 60-day-old animals on canned, home cooked and raw food diets, respectively, in six separate experiments

EXPERIMENT NO.	DATE OF EXPERIMENT AND DIET	AVERAGE WEIGHT OF ANIMALS	BONE ASH OF TIBIA	PROBABILITY OF NON-SIGNIFICANCE OF DIFFERENCE IN BONE ASH BETWEEN ANIMALS ON CANNED FOODS AND	
				Home cooked foods	Raw foods
7	June 20, 1935	*gm.*	%		
	Canned [1]	92	42.7	0.10	< 0.01
	Home cooked [2]	106	40.9		
	Raw	85	38.9		
8	June 22, 1935				
	Canned [3]	99	43.6	< 0.01	0.018
	Home cooked	103	38.8		
	Raw	99	40.5		
9	Nov. 29, 1935				
	Canned	101	47.5	< 0.01	0.26
	Home cooked	121	42.6		
	Raw	108	44.9		
10	Dec. 9, 1935				
	Canned	104	49.7	0.015	< 0.01
	Home cooked	112	43.0		
	Raw	106	43.4		
11	Jan. 20, 1936				
	Canned	128	51.3	< 0.01	< 0.01
	Home cooked	116	44.8		
	Raw	121	46.7		
12	Jan. 27, 1936				
	Canned	128	51.2	< 0.01	0.04
	Home cooked	131	42.5		
	Raw	130	47.5		

[1] Two of six animals discarded because of severe cold and loss in weight.

[2] Two of six animals died; one apparently of polyneuritis, the other of pneumonia.

[3] One of six animals died apparently of pneumonia.

COMMENTS

It may be said that the data presented in these various tables are of an empirical nature and present no evidence in explanation of the results. The data are, however, voluminous enough to be significant. This is apparent from a statistical treatment by Students method of the difference between the per cent of bone ash of animals receiving canned foods and home cooked market foods, respectively, as one comparison and between canned and raw foods as a second comparison. It is obvious there is no significant difference between the results from feeding home cooked and raw foods respectively. The results of statistical treatment are given in the last column of tables 2 and 3. From this it is apparent that in these experiments involving 240 animals with an average of six and two-thirds animals per diet in each experiment—minimum four, maximum nine—the chance that the results are non-significant is less than one in 100 in sixteen out of twenty-four comparisons, and of the remaining eight it is less than five in 100, a reasonable criterion for probability of significance.

Some plausible explanations of these results can be postulated although some of the theories that can be advanced in explanation would be difficult to put to an actual test. It is hoped, however, that in time further explanatory data may be obtained. Certainly the idea that a certain amount of raw foods is absolutely indispensable to health might well be questioned in the face of such evidence. Probably the advocacy of a complete raw food diet has only come from faddists. To consider this question on a rational basis, it should be borne in mind that each dietary essential is a chemical compound or combination of chemical compounds. If cooking does not alter these to prevent their playing their particular role in nutrition, cooking from this standpoint cannot be decried. Cooking inactivates plant enzymes. Their activity in a ruptured raw plant cell is generally not wholesome That conditions should be such in the digestive tract as to make it wholesome is scarcely to be expected.

In these relatively short experiments there is apparent no consistent difference in weight attained as was evident in the earlier experimental procedure in which the dietary regime was maintained over a considerable number of generations. It must be borne in mind that cooked food is a novelty that does not always make an immediate appeal to these animals. Autopsy of a number of animals revealed the fact that the animals receiving raw foods tended to have enlarged caecum and a greater amount of food in the digestive tract and this would be reflected in the weights particularly of young animals. The so-called 'home cooking' is only mild compared to that required of canned foods for sterilization purposes. The raw foods employed, being market produce, must necessarily have been subjected to more or less storage that enhances the fibrous material and is known not to be favorable to vitamin content, which is also affected more or less by the cooking process. Hence little difference between the raw and home cooked diet is not illogical.

The most apparent effect of cooking foods is on their physical structure and texture. While nutritionists have given great emphasis, by their investigations, to the chemical composition of the diet, clinical experience must be turned to for most of the available evidence on the effect of physical structure and texture. It is believed that there is a great deal in the data presented herewith that conforms with this clinical experience.

For those who are familiar with the absorptive properties of certain vegetable fibers toward calcium, there is a plausible explanation for the better utilization of calcium as a result of thorough cooking. In a diet in which the calcium content is below optimum, thorough cooking if it increases calcium availability should effect a higher percentage of bone ash. To illustrate, peas rather rapidly take calcium out of water when suspended in it. Excessively hard water cannot be used in canning peas because this absorbed calcium causes toughening of the skins. The absorption of calcium by the pea skins is a reversible process and the calcium may again be removed

by salt solution. In other words, the pea skins function similarly to a zeolite water softener. The diets used in these experiments, since they did not include milk, probably did not provide an optimum calcium intake. Any factor affecting calcium utilization would thus readily manifest itself. Since cooking tends to break down vegetable tissue, it would tend to lower the absorptive effect of the vegetable fiber for calcium and thus increase calcium availability.

CONCLUSION

Objective evidence from each of three series of experiments indicates there is no inherent virtue in 'rawness.' Furthermore cooking need not incapacitate nutrient elements. It does have beneficial effects on physical structure and texture of foods and inactivates plant enzymes which are detrimental in many instances in handling foods. In so far as canned foods represent the raw product in their prime, they represent a unique means of distributing perishable foods with their inherent nutrient qualities efficiently conserved. Their vitamin content has previously been dealt with and it is here shown that they afford an efficient form of calcium.

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THE INFLUENCE OF SPECIFIC MINERAL DEFICIENCIES ON THE GROWTH OF BODY AND ORGANS OF THE RAT [1]

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ONE FIGURE

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Previous studies (Swanson and Smith, '32, '34, '36) have shown that strict limitation of inorganic salts in the diet of the albino rat results not only in cessation of the increase in body weight, but also in changes in the growth of specific organs. Thus after 60 to 90 days the heart and spleen weigh less than expected for a normal animal of the same body weight, and the kidney and adrenal glands are heavier than usual. The experimental ration used in these studies was notably poor in calcium, sodium, magnesium and chlorine, whereas the relative proportions of phosphorus and of potassium were high (Smith and Smith, '34). In the present study, the influence of individual elements and groups of elements upon the somatic growth and development of certain organs was determined.

Inasmuch as the salt mixture of Osborne and Mendel ('17) was found to be satisfactory for normal development when

[1] The data forming the basis of this paper were taken from a dissertation submitted by Ercel S. Eppright in partial fulfillment of the requirement for the degree of doctor of philosophy, Yale University, 1936.

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incorporated in the basic salt-poor experimental diet, the amounts of the individual inorganic elements included in the various modifications of the basal diet were patterned after it, due account being taken of the consumption of the energy-yielding portion of the ration. Osborne and Mendel ('18) studied the individual elements in a somewhat similar manner, but the subsequent progress in nutrition justifies a reinvestigation of the problem.

PLAN OF STUDY

Male albino rats from the Connecticut Agricultural Experiment Station strain, weighing 45 gm. ±4 at weaning (21 days old) were allowed free access to an adequate stock ration consisting of modified calf meal [2] (Maynard, '30) and paste food,[3] supplemented daily with lettuce and yeast. If they attained a weight of 120 ± 4 when they were 34 days old ±2, they were selected for the experiment.

The basal diet used in all the groups except one (group V) consisted per 100 gm. of

	gm.
Casein[4]	18
Hydrogenated fat[5]	27
Dextrin[6]	55

Vitamins were provided separately each day by 200 mg. dried yeast, 5 drops cod liver oil, 1 ml. of alcoholic extract of wheat germ, and 2 drops of wheat germ oil.

In the basal diet of group V, low-ash lactalbumin[7] replaced the casein and 160 mg. of liver extract,[8] 1 ml. of alcoholic extract of wheat germ, and 5 drops of cod liver oil comprised the vitamin adjuvants.

[2] Modified by the addition of cod liver oil, 3%.

[3] Whole milk powder, 25%; casein, 25%; wheat germ, 20%; lard, 30%.

[4] Described by Swanson and Smith ('32).

[5] Crisco.

[6] White, commercial.

[7] Dry basis, total nitrogen, 14.2%; calcium, none; phosphorus, 0.14%. The Dry Milk Co.

[8] Eli Lilly liver extract no. 343.

The appetite of the low-salt rat is poorer than that of the normal rat. Its daily food consumption has been determined in previous investigations (Brooke and Smith, '33; Clarke and Smith, '35). In order to eliminate the influence of variations in intake of energy, protein and vitamins of the diets, all animals of the present study were given each day the amount of food indicated by the average intake of the large number of low-salt rats previously studied, according to the following schedule:

Period	*Grams of basal diet given per rat per day*
1– 7 days	7.1
8–14 days	6.4
15–35 days	5.7
35–60 days	5.6

The daily quantities of inorganic elements were for the most part based upon 408 mg. of Osborne and Mendel ('17) salt mixture, since this amount represents the average daily consumption of normal male rats eating ad libitum an adequate synthetic diet containing 4% of the salt mixture, a quantity long considered adequate. Formulas were calculated for each diet and for each period in such a way that the desired amount of inorganic adjuvant was offered daily to each rat together with the desired amount of basal ration. Table 1 shows the various groups, the mineral supplements, and the amounts of the different elements given daily together with the abbreviations by which the groups are hereafter designated.

The method insured a fair degree of uniformity in food consumption throughout the groups. As would be expected, however, some animals did not conform exactly to the schedule. These, when possible, were fed by hand. The animals were given redistilled water, and their caging and care followed closely the suggestions of Smith, Cowgill and Croll ('25).

Since most of the changes produced by the restriction of dietary salts are evident after 60 days on the diet, the present experiments were terminated at that time. The animals were

weighed at 4-day intervals just preceding their regular daily feeding. The weights were averaged and treated statistically where possible. Under ether anesthesia the animals were bled from the abdominal aorta and the organs quickly removed, trimmed, blotted, placed in a covered tared bottle, and weighed as quickly as possible.

TABLE 1

Inorganic elements added to diets

GROUP	MINERAL SUPPLEMENT		MILLIGRAMS SUPPLIED DAILY					
	Salt mixture	Milligrams given daily	Ca	Mg	Na	K	Cl	P
I	O and M[1]	408	50.3	7.2	15.4	80.0	21.9	40.9
II	O and M[2] — NaCl + K	408	99.3	7.2	1.7	7.2	3.9	44.5
III	Ca + P + Mg[3]	176	50.3	7.2	1.4	7.0	3.9	40.9
IV	Ca + P[4]	152	50.3	0.2	1.4	7.0	3.9	40.9
V	$CaCO_3$ (lact.)	126	50.3	?	1.1	5.0	...	4.0(?)
VI	$CaCO_3$ (restrict.)	25.6	10.2	0.2	1.4	7.0	3.9	14.6
VII	NaCl + K[5]	242	0.3	0.2	15.4	63.0	24.7	14.6
VIII	NaCl	35.6	0.3	0.2	15.4	7.0	24.7	14.6
IX	Low-salt	...	0.3	0.2	1.4	7.0	3.9	14.6

[1] Prepared as described in J. Biol. Chem., vol. 32, p. 369 ('17).

[2] Essentially Osborne and Mendel salt mixture with sodium and potassium carbonates and hydrochloric acid omitted; calcium carbonate was increased to 238.1 gm.

[3] 152 gm. calcium-phosphorus salt mixture; 24 gm. magnesium carbonate ($3\,MgCO_3 \cdot Mg(OH_2) \cdot 3\,H_2O$).

[4] Calcium carbonate, 404.4 gm.; phosphoric acid, 309.6 gm. of 85% (180.9 ml. of sp.g. 1.71).

[5] Potassium citrate ($K_2C_6H_5O_7H_2O$), 207.8 gm.; sodium chloride, 35.6 gm.

RESULTS

As previously stated, an effort was made to limit the intake of all groups to the amount of energy-yielding and indispensable organic nutrients consumed by the low-salt animals (group IX); these on the average eat approximately half the total amount of food consumed by rats allowed free access to an adequate synthetic diet. The influence of the different salts on the acceptance of the diet was striking. Sodium chloride failed to render the low-salt ration more attractive. Contrary

to expectations, according to the theory of Bunge, reinforcement of the sodium chloride supplement with potassium did not increase the appetite. On the other hand, when calcium was added to the casein diet, either alone, as the carbonate, or with phosphorus, prompt and complete food consumption resulted. Either 10 or 50 mg. of calcium proved sufficient under these experimental conditions. At least a small amount

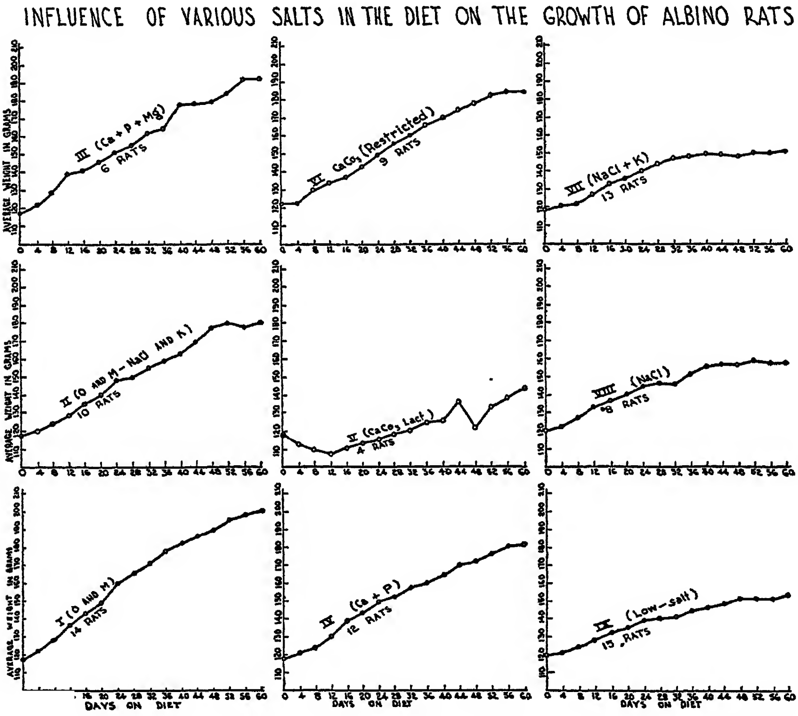

Figure 1

of phosphorus was apparently essential, for when 50 mg. of calcium were ingested daily in the phosphorus-poor lactalbumin low-salt diet, the rats consumed their food less readily.

Growth of animals. Figure 1 shows the average growth curves of the groups. When the total ash constituents of the diet were reduced to 25 mg. per day, growth was markedly retarded, an observation which corroborates that of previous

investigations (Swanson and Smith, '34). Eight days of such rigid restrictions were sufficient to produce a statistically significant difference in body weight as compared with animals similarly limited as to calories and essential protein adjuvants but ingesting daily 408 mg. of the Osborne and Mendel salt mixture (group I). This difference increased until at the end of 60 days the low-salt rats (group IX) had made on the average a gain only 41% of that of group I.

TABLE 2

Weights of organs on low-salt and replacement diets—averages in grams

GROUPS	I	II	III	IV	VI	VII	VIII	IX
ORGAN	O and M	O and M − NaCl + K	Ca + P + Mg	Ca + P	$CaCO_3$ (restrict.)	NaCl + K	NaCl	Low-salt
Liver	5.720	5.179	5.221	5.014	5.044	5.837	5.876	5.543
Kidney	1.275	1.269	1.226	1.328	1.315	1.412	1.723	1.338
Lung	1.067	0.879	0.862	0.872	0.943	0.835	0.962	0.820
Spleen	0.347	0.306	0.310	0.328	0.312	0.344	0.294	0.279
Heart	0.656	0.605	0.615	0.572	0.595	0.621	0.664	0.597
Thymus	0.175	0.184	0.179	0.165	....	0.089	....	0.101
Thyroid	0.014	0.015	0.013	0.015	....	0.014	0.013	0.013
Pituitary	0.0057	0.0051	0.0054	0.0054	....	0.0053	0.0055	0.0054
Body weight	200	180	192	180	184	153	155	152
Number of rats	12	10	6	12	9	12	8	13

The addition of 35 mg. of sodium chloride to the casein low-salt diet (group VIII) did not produce growth significantly greater than that of the animals given the unsupplemented low-salt diet. The addition of 65 mg. of potassium as the citrate to the sodium chloride supplement (group VII) produced such variable results that the average can scarcely be taken as representative of the group; the maximum weight reached after 60 days on this diet was much less than that of group I (Osborne-Mendel salts), and the averages at the various weighing periods were at no time significantly greater than those of the low-salt rats (group IX). Conversely, as was shown by group II, maximum growth was impossible in the absence of added sodium, potassium and chlorine. Evidently, 1.4 mg. sodium and 7.0 mg. potassium, the quantities

of each found in the daily allowance of the basal low-salt diet, are suboptimal for growth. Nevertheless, when other ions were withheld from the diet, the addition of these elements in the quantities normally consumed by rats had no favorable influence on growth.

When calcium carbonate (50 mg. calcium) was added to the lactalbumin low-salt diet as herein described, growth was not appreciably increased. However, when as little as 10 mg. of calcium (as the carbonate) was added to the casein low-salt diet good growth ensued. In all of the groups given diets supplemented with calcium and phosphorus the rate of growth was greatly improved. After 16 days on the diet of group IV

TABLE 3

Summary of organ weights expressed in per cent of body weight—averages

GROUPS		KIDNEY	LIVER	HEART	LUNG	SPLEEN	THYMUS
I	O and M	0.64	2.88	0.33	0.53	0.17	0.086
II	O and M — NaCl + K	0.70	2.90	0.33	0.49	0.17	0.099
III	Ca + P + Mg	0.63	2.72	0.32	0.44	0.16	0.092
IV	Ca + P	0.73	2.77	0.31	0.48	0.18	0.082
VII	NaCl + K	0.74	3.94	0.40	0.55	0.19	0.078
VIII	NaCl	1.08	3.79	0.42	0.62	0.19	....
IX	Low-salt	0.89	3.68	0.39	0.54	0.18	0.067

(calcium and phosphorus), the animals were significantly larger than the low-salt animals, but after 24 days they were significantly smaller than the animals given the complete salt mixture. This intermediate position of the growth curve of group IV between group I and group IX was maintained until the end of the experiment, and ultimately it appeared that calcium and phosphorus alone were jointly capable of supporting approximately 75% of the growth made on the complete salt mixture under these experimental conditions.

In group III, which differed from group IV only in the addition of magnesium, growth was slightly better than in group IV; however, the limited number of rats in the group prevents conclusions regarding the beneficial nature of this quantity of magnesium. While the magnesium requirement

of the albino rat has not been determined, it is likely from the present data that the amount of this element (0.018 milli-equivalents per day) in the basal low-salt diet is insufficient for growth. Osborne and Mendel ('18) observed fair growth on approximately 0.05 milli-equivalent of magnesium per day, whereas Leroy ('26) observed nutritional failure on approximately 0.004 milli-equivalents per day.

Growth of organs. The low-salt diet alters the growth of certain organs; it is stimulating to some while it retards the development of others. After 90 days on the diet the kidneys and adrenal glands hypertrophy, whereas the spleen and heart regress (Swanson and Smith, '36). In the present study the influence of the low-salt diet on the size of these and other organs has been reinvestigated and, in addition, the effects produced by the replacement of specific salts and groups of salts have been observed. Inasmuch as the growth of some organs bears a close correlation to growth of the body as a whole, while others do not, the results here obtained were reported both as absolute fresh weight and in relation to body weight.

The livers of the low-salt rats were grossly not significantly different in size from the livers of rats given all of the salts. The replacement of calcium and phosphorus in the diet (as in groups II and IV) resulted in significantly smaller livers than those of group I. With the replacement of sodium chloride or sodium chloride plus potassium, the average gross weights of the liver at 60 days were as large as those of animals ingesting all the salts.

The growth of the liver bears a fairly close correlation to the growth of the body as a whole, the coefficient as given by Donaldson ('24) being 0.83. In groups II and IV, although the livers were grossly small, the proportion of hepatic tissue to body weight was practically normal. However, in groups VII, VIII and IX the rate of increase in hepatic tissue was greater than that of the body as a whole.

The actual weights of the kidneys were extremely variable in all groups except group I. In the low-salt and most of the

replacement groups, these organs were somewhat larger than normal, but the differences were not statistically significant. In group VIII (NaCl) in which there was unmistakable renal and bladder involvement, some of the kidneys were extremely large, weighing as much as 3.11 gm. per pair. In the kidneys of group IV, which were slightly but not significantly large, histological examination revealed numerous calcium casts. This cast formation which was similar to that induced by parathormone administration was apparently induced by normal amounts of calcium and phosphorus but in the absence of other salts in the diet.[9]

In groups VII (NaCl plus K), VIII (NaCl) and IX (low-salt), the proportion of renal weight to body weight was markedly disturbed. The animals of these groups, after 60 days on the experimental diet, possessed renal tissue 15.6%, 69.0% and 36.8%, respectively, greater per gram of body weight than the average of the animals of group I (Osborne-Mendel salts). The kidneys of rats thus fed continued to grow despite the cessation of body growth.

In all of the groups in which no sodium, potassium, or chloride were added to the basal diet (i.e., groups II, III, IV and IX), the hearts were grossly smaller on the average than those of group I (Osborne-Mendel salts). With these elements supplied (groups VII and VIII) the hearts were not appreciably different in size from those of group I. In all the groups amenable to statistical treatment, these observations were valid. Sodium chloride, with or without potassium, was sufficient to stimulate growth of the heart comparable to that of animals given all the salts and was capable of sustaining such growth independent of increase in body weight.

The only significant difference with respect to the size of the spleens was found in group IX, the low-salt group; the average gross weight of the spleens of this group was 19.6% less than that of group I (Osborne-Mendel salts). In relation to body weight, the splenic tissue of the low-salt rats was not

[9] We are indebted to Dr. H. A. Weiner of the department of pathology for interpretation of the renal changes.

diminished. Throughout the groups the ratio of splenic tissue to body weight was strikingly constant.

In the low-salt and all of the replacement groups except group VIII (NaCl), the lungs were significantly smaller than those of group I (Osborne-Mendel salts). Related to body weight the pulmonary tissue of the sodium chloride supplemented group VIII (NaCl) was 16.7% greater than that of group I. The pulmonary tissue of the other groups retained an approximately uniform proportionality to body weight.

The thymus glands of albino rats maintained for 60 days on the low-salt diet (group IX) were significantly smaller than those of animals given all the salts (group I). The addition of sodium, potassium and chlorine (group VII) contributed nothing toward the growth of the thymus gland; in fact the thymus glands in this group were small and different in form, color and general appearance from the glands of the animals in better condition. In the rats consuming the rations with calcium and phosphorus, the thymus glands were like those of the animals in group I in size; the presence or absence of sodium and/or potassium was without influence. Despite the stunted somatic growth, the thymus tissue per unit of body weight of the low-salt rats (group IX) was less than that of group I (Osborne-Mendel salts) or of group IV (Ca plus P). This stimulation of growth of the thymus gland by calcium and phosphorus in the diet is of interest in connection with the suggestions that this organ is related to calcium metabolism (Zwarenstein, '34).

None of the modifications of the mineral content of the diet produced significant changes in the pituitary glands when fresh weight was taken as the criterion.

While the thyroid glands of the rats of group II and group IV, both of which were supplemented with calcium, weighed slightly more than those of the other groups, the enlargement was not significant. In the present series no significant changes in the thyroid glands were observed.

Because of the frequently suggested relationship between the adrenal glands and mineral metabolism a special study

was made of these organs in a larger series of replacement experiments than herein reported. The data thus secured will be presented in a subsequent paper.

Other changes in tissues. Usually the hair of rats given the casein low-salt diet begins to fall until, after a month on the ration, the abdominal area and legs may become almost bare. The remainder of the coat is unkempt and lusterless. The replacement of sodium chloride (group VIII) or sodium chloride plus potassium (group VII) in the diet did not prevent the loss of hair. However, when the diet included calcium, and phosphorus in addition to that in the casein of the basal ration, loss of hair did not occur. The importance of both elements in this connection is emphasized by the observation that calcium unsupplemented with phosphorus did not prevent loss of hair.

Excitability. Albino rats deprived of salts were in a state of hyperexcitability not nullified by any of the salts given separately. A marked disproportion either of calcium or of potassium in the diet augmented the condition. Toward the end of the experimental period of 60 days, members of group VII (sodium chloride plus potassium replaced) were frequently found in a state of tetany.

Hyperemia. The five rats of group V, which were given daily doses of 126 mg. of calcium carbonate as the only inorganic supplement to the lactalbumin low-salt diet, after 24 to 30 days on the diet, developed a redness of the ears which persisted for about 7 days and then practically disappeared, leaving the ears scabby and pale. These evidences of capillary disturbances were apparent in other parts of the body. The condition was strikingly similar to that described by Kruse, Orent and McCollum ('32) in rats given a diet very low in magnesium. The animals were somewhat excitable, but the extreme nervousness and spasticity described by these investigators were observed in only two members of the group.

In only one other group was this same condition observed even to a limited extent; four of the twelve rats given calcium

and phosphorus (group IV) developed hyperemia on the ears, and scabby areas found in the skin suggested capillary disturbances similar to those occurring in the ears.

Sooner or later during the period of low-salt feeding, the animals show dried blood around the nose. This condition is not correlated with lesions in the lungs but seems to be associated with capillary fragility in the nasal or tracheal mucosa. It was particularly noticeable that this condition was absent in the rats of group IV, given calcium and phosphorus.

The amount of magnesium ingested daily by the animals of group V is not known; in group IV, as was previously stated, 0.2 mg. comprised the daily intake. The diets used by Kruse, Orent and McCollum ('32) contained only 1.8 parts magnesium per million. The fact that the hyperemia occurred in only a limited number of group IV may indicate that 0.2 mg. is near the minimum requirement. Moreover, the fact that the hyperemia did not occur in groups VI, VII and IX which were likewise poor in magnesium but were also poor in calcium may provide further evidence that the calcium-magnesium ratio plays an important role in the etiology of the syndrome.

SUMMARY

With the intake of food calories limited to approximately one-half the usual consumption, calcium and phosphorus are the most effective mineral supplements used in this study from the point of view of increase in body weight, maintenance of size of thymus, and general nutritive well-being. Although sodium and potassium, given separately or together, fail to promote growth in the absence of the remainder of the elements of the Osborne and Mendel salt mixture, their presence is required to support the maximum development possible on the given energy and protein allowance. In the groups consuming rations without calcium but with sodium and/or potassium, the ratios of both heart weight and liver weight to body weight were elevated.

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GROWTH ON HISTIDINE AND LYSINE ADMINISTERED BY SUBCUTANEOUS OR INTRAPERITONEAL INJECTION [1]

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TWO FIGURES

(Received for publication December 23, 1936)

It is well established that the dietary lack of certain amino acids results in cessation of growth, presumably because the body requires them for tissue building or for other normal functions and is unable to supply them by synthesis. If this thesis is correct, growth should again occur when the deficiency is met; the mode of administering the required amino acid should enter into the consideration only insofar as it might influence the rate of supply to the tissues. In other words, the same qualitative response in growth should be obtained whether the amino acid is given by mouth or otherwise; on the other hand, quantitative differences might reasonably be expected unless the methods of administration insured the same continuity and uniformity in distribution of the amino acid.

Alcock ('36) has recently questioned the assumption that preformed amino acids are used for tissue synthesis. He has based his doubt largely on his earlier finding ('34) that tryptophane administered subcutaneously for 15 days to two

[1] The experimental data are taken from a dissertation submitted by Ralph M. Conrad in June, 1936, in partial fulfillment of the requirements for the degree of doctor of philosophy in biochemistry in the Graduate College of the State University of Iowa. The data were presented in abstract before the Iowa Academy of Science at Iowa City, Iowa, April 3, 1936.

animals on a tryptophane-deficient diet did not improve their nutritive condition. Unfortunately, however, his experimental data do not seem adequate to justify his far-reaching assumption ('36) or even to refute prior contrary evidence.

Thus far the effectiveness of administering essential amino acids by injection has been tested only on tryptophane. The earliest such study was made by Jackson ('27) on two animals, each of which was given 25 mg. of tryptophane once daily over a 12-day period. The data show that although the animals did not grow, neither did they undergo as rapid loss in weight as the control animal injected with water. Shichiri and Sakata ('33) gave extremely small doses of tryptophane (1 mg. every other day, followed by 1, 2 and 4 mg. daily) over a long period of time. The three animals thus treated lived until the termination of the study, approximately 120 days after they had been shifted from a casein diet to a deficient diet thus supplemented. All of the animals lost considerably less weight (by 40 to 50 gm.) than the three controls which received no injections. Furthermore, all of the latter died approximately 80 days after the deficient regimen was begun. In their earlier study, du Vigneaud, Sealock and Van Etten ('32) injected a single animal with 10 mg. of tryptophane daily, distributed in four equal doses, for a period of 14 days. They obtained a striking growth response which they were able to duplicate in a second animal on 12 mg. of acetyl-l-tryptophane, similarly administered. The recent and more extensive observations of du Vigneaud, Sealock and Van Etten ('35–'36) fully substantiate their earlier conclusion that tryptophane can be utilized when injected subcutaneously.

Alcock compared the growth of but two animals, each injected subcutaneously with 20 mg. of tryptophane daily in one dose for a period of only 15 days, with the growth of two animals receiving no supplements. Careful measurement of the growth curves indicates that the injected animals actually did grow slightly better than the controls. Furthermore it should be noted that both controls grew slightly during the 15 days, whereas on a preliminary test of the same diet, four

out of six rats lost weight during a 20-day period and five of the six showed loss when the period was extended to 40 days. The differences in growth of the two experimental animals and the two controls cannot in themselves be considered adequately positive, but we cannot agree with Alcock ('36) that the result was 'definitely negative.' In our opinion the data suggest that the test period was not sufficiently long to demonstrate utilization of tryptophane thus administered. Certainly the results were not sufficiently striking in the face of contrary evidence to serve as one of the premises upon which to base a revolutionary hypothesis.

Two significant variations are to be noted in the several studies. The experimental animals of du Vigneaud, Sealock and Van Etten weighed much less (57 to 98 gm.), with but two exceptions (115 and 146 gm.), than any of the rats employed in the other studies (130 to 187 gm.); furthermore, their animals were placed upon a deficient diet for several days before the injections of tryptophane were begun. This procedure is especially important in studies lasting for such short periods as 12 to 15 days. Rats transferred from a stock diet to a synthetic diet, or from a better synthetic diet to a poorer one, frequently lose weight for several days before actually gaining, even though the new diet is adequate. On the other hand, rats which have subsisted for a time on a diet deficient in an essential amino acid usually show prompt growth response when the missing component is supplied.

Because the theoretical implications in these studies involve other essential amino acids, we undertook to test the growth-promoting ability of lysine and histidine when supplied either by subcutaneous or by intraperitoneal injection to rats on diets deficient in one or the other. The data recorded below confirm the view that essential amino acids can be utilized, whether fed or injected.

EXPERIMENTAL

The eighteen young rats used in the histidine studies were all of the same age and weighed between 33 and 56 gm. They

were housed in individual false-bottomed cages and were fed the histidine-deficient diet and vitamin supplements as outlined in table 1. Food consumption and body weights were recorded every 4 days. After an 8-day preliminary period on the deficient diet, all but two animals were given 37 mg. of histidine daily. The histidine was administered as the monohydrochloride in one of three ways: 1) by subcutaneous injection, 2) by intraperitoneal injection, or 3) by incorporation in

TABLE 1

Composition of diets

	HISTIDINE-DEFICIENT DIET [1]	LYSINE-DEFICIENT DIET [1]
	%	%
Histidine-deficient casein hydrolysate [2]	14.5	...
Zein [3]	...	19.5
Tryptophane	0.2	0.2
Cystine	0.3	0.2
Histidine monohydrochloride	...	0.1
Starch	39.5	34.5
Sucrose	15.0	15.0
Hydrogenated cottonseed oil [4]	19.0	19.0
Cod liver oil	5.0	5.0
Salt mixture (Hawk and Oser, '31)	4.5	4.5
Agar	2.0	2.0
	100.0	100.0

[1] The diets were fed ad libitum. Two 100 mg. vitamin B complex tablets (Harris) were supplied separately daily. Distilled water was always available.

[2] Prepared as directed by Conrad and Berg ('37).

[3] For preparation, see Berg ('36).

[4] Crisco.

the vitamin pills. Of the first group, three rats received the daily supplement in two equal doses 12 hours apart and three were given it in a single dose; in the other groups, three animals received two doses and two a single dose daily. The hydrochloric acid was always neutralized by the addition of an equivalent amount of sodium bicarbonate. The solutions for injection were of such strength that the daily supplement was contained in 1 cc. The solutions were sterilized by autoclaving and all reasonable precautions were taken to prevent

infections. The injections were made in different places to prevent unnecessary irritation. The administration of histidine was continued for 32 days; the experiment was terminated 8 days later.

In the lysine studies the same technics were employed. The twenty rats were all of the same age and showed essentially the same range in initial weights (37 to 58 gm.) as those in the histidine series. The composition of the lysine-deficient diet is recorded in table 1. After an 8-day preliminary period on this diet all but two rats received 50 mg. of lysine daily as the dihydrochloride for a period of 32 days. Again exactly enough sodium bicarbonate was added to convert the hydrochloride to the free amino acid. Each of the three types of supplementation was given to six animals, three of which received the lysine in two daily doses and three in one. The study was terminated 12 days after the lysine supplements were discontinued.

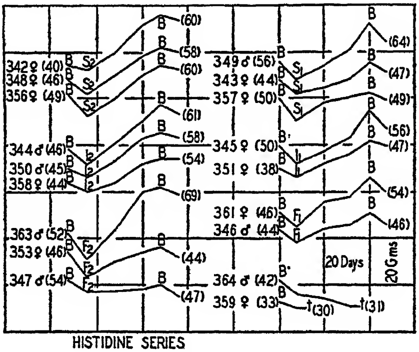
DISCUSSION OF RESULTS

Individual growth curves are presented in figures 1 and 2. In every instance the animals on the histidine-deficient diet lost weight. When the histidine administrations were begun, marked improvement was noted which continued until the supplements were omitted. In every instance both the subcutaneous and intraperitoneal injections induced growth. The data show very clearly that the histidine thus administered was utilized. In fact, growth on histidine supplied by injections compared much more favorably with growth on histidine fed separately than we had anticipated.

These same statements can be made of the lysine series of animals. In every instance differences between growth on the supplemented and unsupplemented zein diets were striking. This was true whether the lysine was injected or given by mouth.

A careful comparison of the several growth curves of the rats receiving two doses of lysine or histidine with those of animals receiving only one slightly favors the former. Individual variations in growth response (table 2) were wide,

however. The use of more animals or an extension of time beyond the 32 days would undoubtedly have minimized these irregularities and made distinctions sharper.



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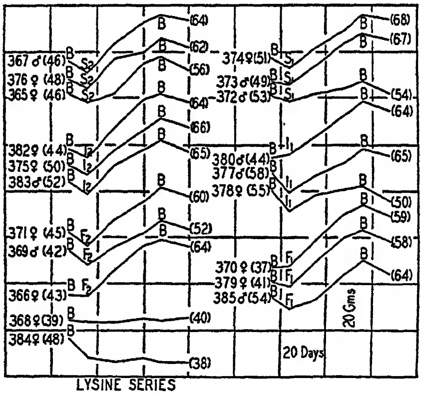

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Figs. 1 and 2 Initial and final weights of the rats are given in parentheses. B represents the deficient basal diet alone. S indicates the supplementation of B by subcutaneous injection of the required amino acid; I, supplementation by intraperitoneal injection; and F, by separate feeding in the vitamin pill. Administration of the supplement in one dose daily is represented by the subscript 1; in two doses daily by subscript 2. The daggers indicate death.

The results which we have obtained are not in harmony with Alcock's ('34) interpretation that injected amino acids do not support growth. They constitute, instead, a refutation of one of his arguments against current concepts of protein synthesis in vivo ('36). In our opinion Alcock failed to ob-

TABLE 2

Average daily growth and food consumption during the 32-day period of amino acid administration

RAT NO. AND SEX	AVERAGE DAILY Gain in weight	AVERAGE DAILY Food consumption	TYPE AND FREQUENCY OF DAILY SUPPLEMENTATION	RAT NO. AND SEX	AVERAGE DAILY Gain in weight	AVERAGE DAILY Food consumption	TYPE AND FREQUENCY OF DAILY SUPPLEMENTATION
	gm.	*gm.*			*gm.*	*gm.*	
Histidine series [1]							
342♀	0.75	3.9	Subcutaneously (twice)	343♀	0.41	3.4	Subcutaneously (once)
348♀	0.66	3.3	Subcutaneously (twice)	349♂	0.75	3.5	Subcutaneously (once)
356♀	0.69	3.4	Subcutaneously (twice)	357♀	0.38	3.2	Subcutaneously (once)
344♂	0.81	3.9	Intraperitoneally (twice)	345♀	0.69	3.5	Intraperitoneally (once)
350♂	0.59	3.0	Intraperitoneally (twice)	351♀	0.47	3.3	Intraperitoneally (once)
358♀	0.44	3.6	Intraperitoneally (twice)				
363♂	0.97	4.0	Per os (twice)	361♀	0.66	3.3	Per os (once)
353♀	0.41	3.6	Per os (twice)	346♂	0.41	3.3	Per os (once)
347♂	0.09	3.2	Per os (twice)				
364♂	—0.18	2.3	None (died on the 28th day of the period)				
Lysine series [2]							
367♂	0.70	4.1	Subcutaneously (twice)	374♀	0.72	4.0	Subcutaneously (once)
376♀	0.69	4.3	Subcutaneously (twice)	373♂	0.69	4.2	Subcutaneously (once)
365♀	0.63	4.1	Subcutaneously (twice)	372♂	0.28	3.2	Subcutaneously (once)
382♀	0.88	4.2	Intraperitoneally (twice)	380♂	0.69	3.8	Intraperitoneally (once)
375♀	0.75	3.9	Intraperitoneally (twice)	377♂	0.56	4.1	Intraperitoneally (once)
383♂	0.72	3.9	Intraperitoneally (twice)	378♀	0.31	3.7	Intraperitoneally (once)
371♀	0.84	3.8	Per os (twice)	370♀	0.81	4.2	Per os (once)
366♀	0.75	4.1	Per os (twice)	379♀	0.75	3.6	Per os (once)
369♂	0.63	3.6	Per os (twice)	385♂	0.66	4.0	Per os (once)
384♀	0.06	2.6	None				
368♀	0.03	3.0	None				

[1] During the initial 8 days, animals in the histidine series lost 0.71 gm. per day on the average and ingested 3.1 gm. of food per day; during the 8-day after period corresponding average figures were 0.51 gm. and 3.3 gm., respectively, for all rats which had previously received histidine.

[2] Losses for the animals of the lysine series during the initial 8 days averaged 0.51 gm. per day; during the final 12 days 0.36 gm. Average daily food consumption figures for the two respective periods were 3.5 gm. and 3.9 gm.

tain definitely positive results with tryptophane largely because his test period, as conducted, was too short. Had the injections followed a period of deficiency, as in the case of the present histidine and lysine studies, a more immediate response might have occurred. Incidentally, feeding an essential amino acid in an intimate mixture with other dietary components (Alcock, '34) cannot be considered a suitable control for a regimen in which the essential amino acid is administered by injection in one or two doses. The two regimens are not analogous. We have partially overcome this objection in the present studies (as indeed du Vigneaud, Sealock and Van Etten did in theirs, '36) by incorporating the amino acid in the separate vitamin B supplement. This procedure is not ideal, but it permits a much fairer comparison and is practical.

In our several studies on tryptophane, lysine and histidine metabolism, we have invariably obtained better growth by incorporating these amino acids in the otherwise deficient diet than by feeding them separately in the vitamin pills. In fact, significant variations in growth are induced, even when the same quantity of tryptophane is fed separately, by altering the frequence of dosage (Berg and Rose, '29). These observations are reasonable and in accord with the accepted theory that an essential amino acid cannot be utilized efficiently for tissue synthesis unless the other amino acids needed in the construction are also present in adequate amounts. The administration of one or two relatively massive doses each day would naturally provide a great excess at one time and a shortage at others.

CONCLUSIONS

Both histidine and lysine can be utilized effectively for growth when administered to rats either by subcutaneous or by intraperitoneal injection. The rate of growth of such animals compares favorably with that of other animals fed the same amino acids separately. These observations are entirely in accord with currently accepted theories of protein metabolism.

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ON THE CLAIM FOR A NEW ESSENTIAL DIETARY FACTOR IN MAMMALIAN LIVER [1]

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TWO FIGURES

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Elvehjem and co-workers ('35, '36 a, '36 b) and Koehn and Elvehjem ('36) have reported a new dietary factor from liver which they find to be necessary in addition to vitamins $B(B_1)$ flavin, B_4, B_6 and 'B_2' [which, it would seem, is identical with the 'filtrate factor' of Lepkovsky and Jukes ('35, '36 a, '36 b) and Lepkovsky, Jukes and Krause ('36)] for growth of rats. We wish to report certain results which we have obtained with the alcohol-ether precipitate from air-dried fresh hog liver, following the procedure described by these authors. From the supernatant liquid we prepared an adsorbate with the resulting filtrate. A water extract of the precipitate showed no fluorescence when tested by the 'black light' (Supplee and others, '36), but the supernatant liquid was highly fluorescent.

EXPERIMENTAL

Two types of basal diets have been used in this laboratory in tests for flavin and vitamin B_6 (Halliday and Evans, '37). Diet 787-B has the following composition: casein 18%, salts 4%, filtered butter fat 8%, cod liver oil 2% and cornstarch

[1] Aided by grants from the Board of Research and the College of Agriculture of the University of California, and the Rockefeller Foundation of New York.
The following materials were generously contributed: Cod liver oil by E. R. Squibb and Sons; vitamin B_1 concentrate by Merck & Co., through the courtesy of Dr. R. E. Gruber; Lactoflavin by the Vitab Products Company, through the courtesy of Mr. G. F. Siemers; fresh hog liver by H. Moffat Company, San Francisco, through the courtesy of Mr. F. M. Kleppe.

68%. In diet 793 the butter fat is reduced to 3% and 73% sucrose is included in the place of cornstarch. The casein is extracted with boiling 95% alcohol until no fluorescence can be detected when the alcohol extract is examined by a 'black light' (four extractions). This procedure is followed by two extractions with cold 60% alcohol and one with cold 95% alcohol.

Twenty-one-day-old female rats weighing 40 to 50 gm. were given the basal diet, supplemented by 200 γ of a potent vitamin B concentrate until the weight was stationary and mild dermatitis developed. This required about 4 to 5 weeks with diet 787-B, and 3 to 4 weeks with diet 793. With the latter diet, rats receiving B or B and flavin, with or without a supplement low in B_6, survived about 65 days from weaning with 100% incidence of severe dermatitis. With diet 787-B only three out of a group of eighty rats receiving little or no vitamin B_6 failed to develop dermatitis. Survival periods averaged about 100 days from weaning for the animals which developed dermatitis.

In the first experiments here reported we used diet 787-B. Using litter mates we tested the dried liver, and the alcohol-ether precipitate, the adsorbate and the filtrate alone or in combination, without added flavin. Figure 1 A shows the results. The experiment represented by curve IX was carried out somewhat later, but is included in this group for comparison. These rats received diet 793 supplemented with vitamin B, flavin, and the precipitate.

All rats which received the liver precipitate were completely protected from dermatitis. When the precipitate was fed alone there was little gain, but when supplemented with flavin or the adsorbate and filtrate obtained from the supernatant liquid, the rats gained 8 to 10 gm./ week. Animals receiving dried liver in an amount equivalent to 0.25 gm. showed slight lesions, but those on the higher level were in excellent condition. The remaining rats developed severe dermatitis.

We have prepared a flavin concentrate from fresh hog liver. The filtrate (filtrate W) remaining after the adsorption in this process is comparatively low in vitamin B_6, but it carries the

'filtrate factor.' When this filtrate is fed to rats receiving vitamin B and flavin, there is some growth, but the animals develop mild dermatitis. Addition of the liver precipitate in an amount equivalent to 1.0 gm. fresh liver induced rapid healing and a gain of 1 to 1.5 gm. per day (curve XI, fig. 1 B). These animals were receiving diet 793, with 200 γ daily of vitamin B concentrate.

As a final rather crucial test we gave the precipitate in a curative experiment. Out of forty rats which had received various materials in tests for their vitamin B_6 value, only seven had survived an 8-week experimental period. All had

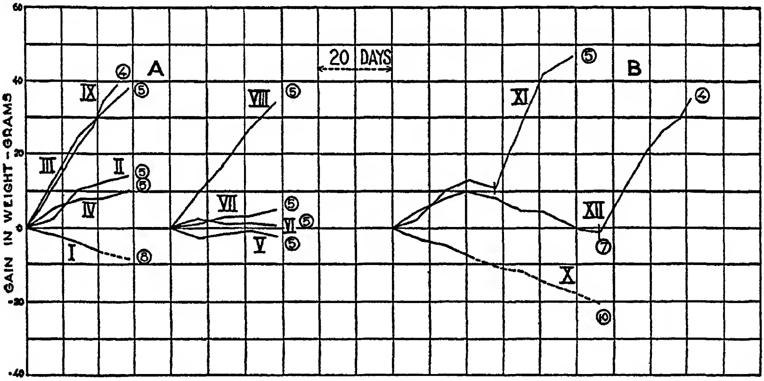

Fig. 1 A, curves representing average growth of rats which received diet 787-B plus 200 γ of vitamin B_1 with I, no additional supplement; II and III, dried liver equivalent to 0.25 and 0.5 gm. fresh material; IV, alcohol-ether precipitate equivalent to 1.0 gm. fresh liver; V, adsorbate from supernatant liquid equivalent to 1.0 gm. fresh liver; VI, filtrate resulting after adsorption equivalent to 1.0 gm. fresh liver; VII, the adsorbate and filtrate in combination equivalent to 1.0 gm. fresh liver each; VIII, adsorbate and filtrate supplemented by the precipitate equivalent to 1.0 gm. fresh liver each; IX, curve representing average growth of rats receiving diet 793 plus vitamin B_1, 5 γ crystalline lactoflavin, and the liver precipitate equivalent to 1.0 gm. fresh material.

B, curves representing average growth of rats which received diet 793 plus 200 γ of vitamin B_1 and X, no additional supplement; XI, filtrate W equivalent to 3.0 gm. fresh liver and 5 γ lactoflavin and at | the ether-alcohol liver precipitate equivalent to 1.0 gm. was added; XII, 5 γ lactoflavin and various materials under test (as described in text). At | the alcohol-ether liver precipitate was substituted for the test material. Dotted line indicates that one (or more) rats have died. The curve from this point represents growth of surviving animals. The numeral at the end of each curve indicates the number of rats of which results are included in the curve.

severe dermatitis, the ears were swollen and sore, there was a large raw area around the nose and mouth, the feet and forelegs were edematous and sore, and there was diarrhoea and a bloody urine. The rats had received vitamin B and flavin as well as the supplementary material in addition to diet 793. At the end of the experimental period the liver precipitate was substituted for the material which had been under test. Although these seven rats had received various supplements, their growth curves were similar and are averaged together. Of the group, three were too ill to respond and died within a few days after the precipitate was given, but the remaining four were rapidly cured and showed a gain of about 1.5 gm. per day (curve XII, fig. 1 B).

From these experiments we would conclude that the precipitate carries vitamin B_6. It is known that this vitamin can be adsorbed. It appears to be necessary to use a rather large amount of fuller's earth and a rather acid medium. The liver precipitate comes down as a mass of very fine particles, and it seems reasonable at least to assume adsorption of the vitamin on this material.

DISCUSSION

It is somewhat difficult to correlate these results with those of Elvehjem and co-workers. These authors state that rats which received their various basal diets supplemented by vitamin B only or by B, flavin, and 'B_2' showed no symptoms which might aid in determining the specific deficiencies. Since rats receiving our basal diets (particularly those which included sugar in place of starch) supplemented with B and flavin, uniformly developed dermatitis, specific of vitamin B_6 deficiency, it might seem that the diets used by these authors carried some vitamin B_6. In later work they included 12% white corn to provide vitamins B_4 and B_6, and in those diets dextrin was used in place of sugar. Here again there was no gain in weight, when supplemented by vitamin B, flavin and 'B_2,' and the animals were reported to have shown no symptoms other than lack of growth and general emaciation. We have tested the vitamin B_6 value of ground whole wheat,

but not corn. When rats received diet 793 supplemented by vitamin B and flavin, the addition of 1.0 gm. per day of whole wheat induced a gain averaging 0.6 gm. daily. It was not possible to obtain food consumption records with a sugar diet, but rats receiving a cornstarch diet consumed about 6 gm. daily when making this rate of gain. If a corresponding consumption of the sugar diet is assumed, we would have a level of 14% whole wheat in the diet. Since Elvehjem and coworkers included white corn at 12% and used dextrin as the carbohydrate, the results should be comparable. In our hands even a whole wheat extract, prepared according to the Bour-

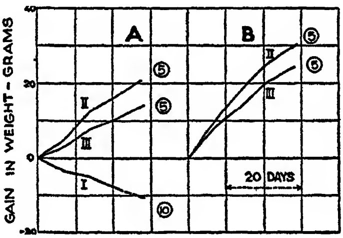

Fig. 2 A, curves representing average growth of rats which received diet 793 with 200 γ of vitamin B_1 daily and I, no additional supplement or B_1 and 5 γ of lactoflavin; II, 1.0 gm. daily of whole wheat plus 5 γ of lactoflavin; III, whole wheat extract equivalent to 4.0 gm. whole wheat daily and 5 γ of lactoflavin.

B, curves representing average growth of these rats when plotted as gain above that of litter mates receiving vitamin B_1 or B_1 and lactoflavin.

Dotted line indicates that one (or more) rats have died. The curve from this point represents growth of surviving animals. The numeral at the end of each curve indicates the number of rats of which results are included in the curve.

quin-Sherman ('31) method, induced a gain of about 0.5 gm. per day when fed in amounts equivalent to 4.0 gm. of whole wheat and supplemented by vitamin B and flavin (fig. 2).

Differences in the diet fed to stock rats in different laboratories will undoubtedly lead to different results in the experimental animals. However, table 1 gives results obtained by Copping ('36), by Birch, György and Harris ('35) and ourselves when white or yellow corn, whole wheat, or whole wheat extract are fed to rats receiving diets deficient in the B vitamins, and supplemented with crystalline or highly purified concentrate of vitamin B and with pure flavin. These are

TABLE 1

Comparisons of results from various laboratories from tests for the vitamin B_6 potency of whole wheat and corn

RESULTS REPORTED BY	CARBOHYDRATE IN BASAL DIET	SUPPLEMENT TO DIET	TEST MATERIAL	AVERAGE WEEKLY GAIN, GM.	RESULT
Copping	Maize sugar	Peters B_1 concentrate and 12 γ hepaflavin	Alcohol extract of maize or wheat 4.0 gm.	11.5	Perfect cure of florid dermatitis
Birch, György and Harris	Rice starch	Crystalline or highly purified B_1 and 10 γ lactoflavin	Maize, yellow		
			5.0 gm.	17.0	Perfect cure of florid dermatitis
			3.0 gm.	14.0	Perfect cure of florid dermatitis
			2.0 gm.	9.0	Perfect cure of florid dermatitis
			1.0 gm.	7.0	Perfect cure of florid dermatitis
			0.75 gm.	6.0	Perfect cure of florid dermatitis
			Maize, white		
			2.0 gm.	8.0	Perfect cure of florid dermatitis
			1.0 gm.	7.0	Perfect cure of florid dermatitis
			0.5 gm.	2.0	Perfect cure of florid dermatitis
			Whole wheat (ground)		
			3.0 gm.	12.0	Perfect cure of florid dermatitis
			2.0 gm.	12.0	Perfect cure of florid dermatitis
			1.5 gm.	8.0	Perfect cure of florid dermatitis
			1.0 gm.	4.0	Inconstant
Present authors	Sucrose	200 γ vitamin B_1 concentrate with 5 γ lactoflavin	1.0 gm.	4.2	No dermatitis
			Alcohol extract of whole wheat $\approx$ 4.0	3.0	No dermatitis
Elvehjem and co-workers	Dextrin	B_1 concentrate, flavin and 'B_2'	12% white corn in diet	....	Protection from dermatitis. No gain in weight

compared with the results of Elvehjem and co-workers when 12% white corn is included in the diet, and vitamin B, flavin and 'B_2' are fed.

In the experiment reported by Copping, the rats were depleted up to 14 weeks, and had practically maintained their weight. Birch, György and Harris stated that about 7 weeks were required to induce dermatitis in animals. In our experiments, the rats developed symptoms in 3 to 4 weeks, and unless supplemented, died on an average of 9 to 10 weeks after weaning, with an average loss of 10 to 20 gm. in weight. The gain of rats receiving daily 1 gm. of whole wheat or whole wheat extract equivalent to 4 gm., above that of litter mate controls, receiving vitamin B or B and flavin, averaged 7.0 and 6.0 gm. per week, respectively, thus falling between the results from the other two laboratories. As seen in table 1 the results of Elvehjem and co-workers are very different.

From our results it appears that the cure of dermatitis in the rats resulting from feeding the liver precipitate was due to vitamin B_6 in this material. Whether the excellent rate of gain induced by the precipitate when fed with vitamin B and flavin or adsorbate (with or without the liver filtrate) was due to a new factor or to the combined action of vitamin B_6, flavin and the 'filtrate factor,' further work alone will show. In the experiments represented by curves IX and XII (fig. 1), no 'filtrate factor' was provided. However, in the first case the rats had been subjected to only a 24-day depletion period before the additional supplement was fed. In the second, the rats had been receiving various fractions from liver filtrate W prepared in an attempt to concentrate vitamin B_6. In both groups the body store of the 'filtrate factor' was undoubtedly sufficient to allow growth. This is confirmed by curve XI (fig. 1) where a similar rate of gain is obtained when filtrate W was fed with the precipitate and flavin. Also since we fed the crude precipitate without further purification, it seems probable that some 'filtrate factor' may have been present in the material.

From the liver we used and the procedure followed, it would seem that we obtained a fairly clear separation of flavin and

vitamin B_6. From the results of Elvehjem and co-workers it appears that some, at least, of the original liver flavin had been adsorbed by the precipitate, since rapid growth resulted from feeding the precipitate as the sole supplement to their white corn diet.

The lack of growth obtained by Elvehjem and co-workers when the white corn diet was supplemented with flavin and 'B_2' is difficult to understand, unless the flavin was not given at a sufficiently high level. In our work we used crystalline lactoflavin, as did also the English workers.

It is also difficult to understand why the rats receiving their various basal diets (without corn) should have died without showing symptoms of vitamin B_6 deficiency if the vitamin B concentrate was adequate. György ('35) found that too low a dose of vitamin B delayed the onset of vitamin B_6 symptoms. We tested each lot of vitamin B concentrate and fed it at the level which would induce a gain of at least 1 to 1.5 gm./day in rats receiving the Chase and Sherman ('31) vitamin B deficient diet.

The difference in results may be due to biological variations in the experimental animals. If such is the case, it will be difficult for one laboratory to confirm results obtained in another.

CONCLUSIONS

Experiments were carried out in an attempt to confirm the results of Elvehjem and co-workers regarding the new essential dietary factor found in mammalian liver. Our results appear to indicate that the alcohol-ether precipitation procedure carries down vitamin B_6, since the precipitate is highly effective in preventing and curing severe dermatitis.

We appear to obtain a separation of flavin and vitamin B_6 by this method, the flavin passing into the supernatant liquid.

Our results do not demonstrate a new factor essential in addition to vitamins B (B_1), flavin, B_4, B_6 and 'B_2' as postulated by these workers. However, until or unless results obtained in one laboratory can be duplicated in another, it is difficult to draw absolute conclusions.

ADDENDUM

A copy of this manuscript has been submitted to Professor Elvehjem. In a personal communication he states in part, "The results you have tabulated interest me very much because they merely add more evidence to our conclusion that liver contains a new essential dietary factor." His interpretation is as follows:

Our rations were developed with the idea of producing a vitamin B_6 deficiency. Our casein was lower in vitamin B_6 and higher in the alcohol precipitate factor than his. Due to this fact we produced more severe vitamin B_6 deficiency in a shorter time than most other laboratories.

Our results with air dried liver check those of Birch, György and Harris. These authors, however, found that it required an amount of liver extract equivalent to more than 5.0 gm. of fresh liver to equal 1 'rat day dose' of vitamin B_6. He emphasizes the fact that they (Elvehjem and co-workers) worked with liver extract. When this extract was fed at a level of 5% ($\Bumpeq$ 6.0 gm. fresh liver daily) with a sucrose diet, excellent growth resulted. With a 2% level ($\Bumpeq$ 2.4 gm.) the rats manifested symptoms of vitamin B_4 and B_6 deficiencies. For this reason they included 12% white corn. With liver extract $\Bumpeq$ 2.4 gm., rats gained 100 gm. in 30 days whereas our results show a gain of only 40 gm. in 30 days when rats received 0.5 gm. fresh liver daily. (In experiments not included in this paper we obtained a gain of approximately 53 gm. in 30 days when 1.0 gm. equivalent of liver was fed, which is about half the gain Elvehjem and co-workers obtained with 2.4 gm.) Elvehjem states that obviously 0.5 gm. fresh liver does not supply enough alcohol precipitate factor for optimum growth, whereas liver extract $\Bumpeq$ 2.4 gm. daily does supply enough.

With regard to the gain we obtained with whole wheat extract $\Bumpeq$ 4.0 gm. whole wheat, he states that if our ration had not been low in the alcohol precipitate, the gain should have been 3 gm. per day. He states that, using 12% wheat, his results were similar to those obtained with corn. The higher gains obtained by the English workers he considers

were due to the fact that their rations or the grains were higher in this factor.

He concludes: "The fact that you found the alcohol-ether precipitate fraction from whole liver to be a good source of vitamin B_6 does not invalidate our work on a new factor found in liver extract. However, your work does make a definite addition to our knowledge of vitamin B_6."

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VITAMIN B AND G VALUES OF PEAS AND LIMA BEANS UNDER VARIOUS CONDITIONS [1]

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TWO FIGURES

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Differences in the vitamin B and G values found in the literature for the same food are not surprising if one considers that in making the determinations several technics may have been employed—some doses being protective, others curative, while others are designed to give weight maintenance and still others growth. Furthermore, the total amount of any vitamin present in plant material may depend on such factors as variety, stage of development and conditions of growth. Thus Bell and Mendel ('22) found spring wheat two to three times as rich in the vitamin B complex as winter wheat, while Spohn (Sherman and Smith, '31) reported that two varieties of apple differed as much as 15% in the vitamin B complex, a difference due to a variation in vitamin G rather than in vitamin B (Cornell University Agr. Expt. Sta., '30).

An example of the influence of the stage of development is furnished by Hunt and Krause ('31), who found that early pasture produced milk with a higher content of both of these vitamins than did mature pasture. Eddy, Kohman and Carlsson ('26), comparing several types of canned peas as a source of the vitamin B complex, concluded that the more mature the pea the greater the vitamin content, but since the criterion of maturity was the relative size of the peas, this conclusion

[1] Based on a thesis presented by Esther H. Funnell Phipard in partial fulfillment of the requirements for the degree of doctor of philosophy.

would scarcely seem warranted, for some varieties are small at maturity while others are large.

It is not clear at present to what extent soil and climatic conditions affect the vitamin B and vitamin G content of plants. Rowlands and Wilkinson ('30) found that grass seed from land fertilized with manure was more active in promoting growth in young rats than seed from artificially enriched land. McCarrison and Viswanath ('26) found wheat to be 15% richer in the vitamin B complex when cattle manure had been used than when mineral fertilizer was employed. In contrast to this are the findings of Hunt ('27), who checked the content of the vitamin B complex of wheat grown on soils to which had been added various organic and inorganic fertilizers, and found little difference due to the treatment. There was, however, wide variability in the vitamin B content from year to year, which Hunt ascribed to differences in climatic conditions. Hanning ('36) has recently reported a difference of 130% between the vitamin B content of two samples of canned strained peas grown under similar conditions in 2 different years, but much less difference in the vitamin B values of two samples of carrots and green beans, while beets and spinach were practically the same in the two samples tested.

With regard to the influence of ordinary cooking processes, widely different results have been reported. Vitamin G is known (Smith and Hendrick, '26; Chick and Roscoe, '30; Keenan, Kline, Elvehjem and Hart, '35) to be relatively stable to the temperatures of ordinary cooking processes and therefore no appreciable loss of this factor due to heating alone would be anticipated. Vitamin B, on the other hand, is liable to considerable destruction by heat, the extent depending mainly upon temperature, duration of heating, and hydrogen-ion concentration of the solution. Roscoe ('31) found no loss in cooking with regard to either the vitamin B or vitamin G of carrots; in fact the cooking appeared to increase the availability. Munsell and Kifer ('32) reported a 50% loss in vitamin B in broccoli when cooked for 15

minutes. Dye and Hershey ('28) found a 45% depreciation when peas were blanched 5 minutes and processed 40 minutes compared to one of 25% when blanching was omitted but the processing continued for 40 minutes. Sherman ('32) reported that when fluid milk was heated for 6 hours at 100° C. about one-fourth of the vitamin B was destroyed. In a study by Sherman and Burton ('26) of the influence of hydrogen-ion activity, tomato juice heated for 1 hour at 100° C. was found to lose vitamin B with the increasing alkalinity of the solution.

Little is reported in the literature about the effects of drying and storage on the vitamin B and vitamin G content of peas, beans and other foods which are used in the dried form. Salmon ('26) found a loss of about 50% in the potency of the vitamin B complex of velvet beans stored for 3 years, but could find no such difference in soy beans during a similar period.

One of the newer methods of preservation used for a great variety of foods is quick freezing. By this process fruits and vegetables can be prepared, washed, graded, packed and quickly frozen all within 5 to 6 hours of the time of harvesting. Although a considerable number of studies have been made on the vitamin C values of frozen foods, no reports regarding the effect of this treatment on the vitamin B and vitamin G have as yet appeared.

Another interesting treatment of seeds used extensively by eastern peoples is sprouting. Several varieties of beans are used in this way. The rapid synthesis of vitamins A and C in the germinating seed has been clearly established by studies on a wide variety of materials. Less is known, however, concerning possible changes in the vitamin B and vitamin G content of young seedlings. Miller and Hair ('28) found a small but measurable amount of the vitamin B complex in etiolated mung bean sprouts which had been grown 4 days according to the custom of Orientals living in Hawaii. Since a liberal use of sprouted seeds in the diet will contribute very significant amounts of two important dietary essentials, vitamin A and vitamin C, it would be of interest to know what kind of

contribution of vitamin B and vitamin G such a food could make to the dietary.

The purpose of this study was to determine quantitatively the vitamin B and vitamin G content of two seeds important as foods, the pea (Pisum sativum) and the Lima bean (Phaseolus limenanus), in the green state, when raw, cooked and stored under freezing conditions; also when mature. The effect of sprouting the mature pea was also measured.

The peas used were two common varieties, Thomas Laxton and Alderman and the Lima beans were Henderson Bush, a dwarf variety known as the 'baby Lima' type. Fresh peas were obtained from the 1934 crop grown at Albion, New York, especially for commercial preservation. They were shipped at once after picking and were stored at 38°C. during the experimental period. Moisture determinations made weekly showed very little change, the average being 75.5%. Fresh Lima beans were grown by one of us from a sample of the seed used to produce the frozen ones. The first beans picked were slightly immature and showed a moisture content of 71.3% as compared to an average 65.8% for the later pickings. Since the feeding tests were begun on the younger beans, the amount fed of the more mature ones was adjusted to be equivalent in dry weight to that of the immature beans. The peas and beans as needed were shelled, ground in a food chopper and immediately weighed.

Frozen peas [2] were from the 1933 and 1934 crops and the frozen Lima beans were from the 1934 crop. They were kept in the original frozen packages at a temperature below freezing, a few being removed as needed and dried from 10 to 15 minutes on absorbent paper to remove the extraneous ice particles. Moisture determinations made after this treatment of the peas showed good agreement with those made on raw fresh ones, i.e., an average of 75.5% for both.

The mature pea and Lima bean seeds tested were samples of those used to grow the 1934 crops. Equal quantities of the

[2] The frozen peas, frozen Lima beans, fresh peas and pea and bean seed were kindly furnished by the Birdseye Frosted Foods Co.

two parieties of peas were mixed and then several lots of 50 and 100 seeds each were weighed in order to obtain an average figure for the weight of an individual seed. Both pea and Lima bean seeds were ground and mixed before the portions to be fed were weighed.

Sprouted peas were developed in a greenhouse [3] where the seeds (the same as those tested) were planted in flats of coarse washed sand and allowed to grow for 14 days, at which time the sprouts had attained a height of from 3 to 6 inches. The seedlings were then carefully removed from the flats, counted and washed free of sand and the seed remnants separated from the sprouts and roots. These two types of material, the residue of the seed and the new plant growth, were dried under an electric fan for 2 to 3 hours and then over night in a drying oven at 60°C.

Cooked peas were prepared as follows. A small quantity of the fresh (1934) peas was shelled, weighed and put into enough boiling water barely to cover them. They were cooked 15 minutes from the time they began to boil and were covered most of the time to prevent boiling dry. They were then weighed again and if necessary water was added to bring them to the original weight, so that 1 gm. of cooked peas was equivalent to 1 gm. of the raw material. The cooked peas were mashed with a fork to insure more uniform sampling, and were stored in a covered glass jar at a temperature of about 38°F.

Rats from mothers fed two-thirds whole wheat, one-third whole milk powder and sodium chloride equal to 2% of the weight of the wheat, plus lean beef three times per week, were weaned between the twenty-first and thirtieth day of age, when they weighed from 40 to 50 gm. They were housed individually in metal cages with raised screen bottoms of ½ inch mesh. They had access at all times to distilled water and the basal diet, the consumption of which was recorded for each animal. Supplements were fed three times per week,

[3] Greenhouse facilities were made available through the courtesy of Archibald H. Funnell.

each portion being one-third of the amount required for 7 days. So far as possible equal numbers of males and females were used in each group of animals. Negative controls from nearly every litter were maintained on the basal diet.

VITAMIN B EXPERIMENTS

The basal diet lacking vitamin B was essentially that of Chase and Sherman ('31). It consisted of extracted casein, 18%; Osborne and Mendel salt mixture, 4%; cod liver oil, 2%; butter fat, 8%; cornstarch, 48% and autoclaved yeast, 20%. Two or three rats were kept in one cage until they were depleted, which occurred in an average of 16 days. Although a 4-week test feeding period would have been adequate, one of 5 weeks was selected since the 5-week figures were available. There was no significant difference in the results obtained at 4 and 5 weeks.

Frozen peas of the 1933 crop were fed at three levels, 0.5, 0.75 and 1.0 gm. daily, with resulting average gains for the 5-week period of 0.8, 4.7 and 9.2 gm. per week, respectively. When the fresh peas of the 1934 crop arrived, they were fed at a level of 0.5 gm., and gained on the average 7.2 gm. per week. When the 1934 peas were cooked as previously described and fed the same amount (0.5 gm.), the average gain was 5.3 gm. per week. This would indicate that a loss of 26% had been brought about by the process of cooking.

Since there was a difference between the vitamin B content of frozen peas of the 1933 crop and fresh peas grown in 1934, the latter crop was tested frozen as soon as the 1934 packing became available. With 0.5 gm. of these fed daily the animals showed an average gain of 7.6 gm. per week for the period. Therefore there was no loss of vitamin B due to the freezing process.

The mature pea seeds were fed in portions equivalent on a dry weight basis to the fresh peas fed, 0.13 gm. of ripe seed being equivalent to 0.5 gm. of fresh peas. The twelve rats so fed made an average gain of 4.2 gm. per week. The difference in average gain of 3 gm. per week indicates that the

vitamin B content of the dry mature pea seed was much lower than that of the fresh peas. The results are shown in table 1 and figure 1.

The fresh peas proved to be a rich source of vitamin B, containing approximately 3 Sherman-Chase units per gram.

TABLE 1

A summary of the growth response of animals used to determine the vitamin B content of peas and Lima beans

MATERIAL TESTED	AMOUNT FED DAILY	DRY WEIGHT EQUIVALENT	NUMBER OF RATS	AVERAGE GAIN IN 5 WEEKS	AVERAGE GAIN PER WEEK
	gm.	*gm.*		*gm.*	*gm.*
Frozen peas (1933)	0.50	0.12	7	3.8	0.8
	0.75	0.19	10	23.5	4.7
	1.00	0.25	4	45.8	9.2
Frozen peas (1934)	0.50	0.12	10	38.1	7.6
Fresh peas (1934)	0.50	0.12	10	35.9	7.2
Cooked peas (1934)	0.50	0.12	7	26.3	5.3
Pea seed (1933)	0.13	0.12	12	21.1	4.2
Sprouts and roots of one pea seed	0.09	....	8	22.9[1]	5.7
Seed residue of one pea seed	0.05	....	8	— 7.1[1]	—1.8
Fresh Lima beans (1934)	1.00	0.29	7	35.2	7.0
	0.70	0.20	10	19.5	3.9
Frozen Lima beans	1.00	0.29	3	— 6.3[1]	—1.6
Lima bean seed (1933)	0.31	0.28	9	16.8	3.4

[1] Four weeks.

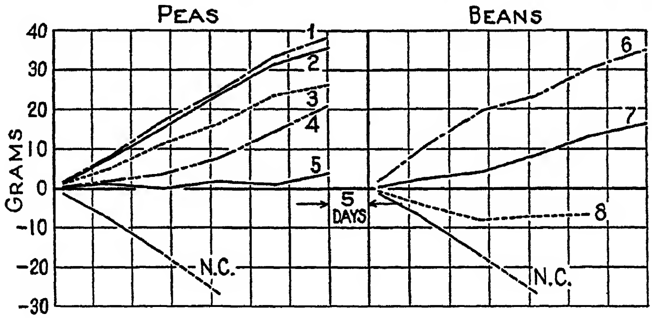

Fig. 1 Growth of animals receiving a vitamin B deficient diet plus daily supplements of the following: 1, 0.5 gm. frozen peas (1934); 2, 0.5 gm. fresh peas (1934); 3, 0.5 gm. fresh peas cooked; 4, 0.13 gm. pea seed equivalent to 0.5 gm. fresh peas; 5, 0.5 gm. frozen peas (1933); 6, 1.0 gm. fresh Lima beans (1934); 7, 0.31 gm. Lima bean seed equivalent to 1.0 gm. fresh Lima beans; 8, 1.0 gm. frozen Lima beans (1934); N.C., negative controls.

This is a higher vitamin B content than most vegetables which have been investigated. The fresh peas were found to be twice as rich in vitamin B as the fresh Lima beans. There was very good agreement between the vitamin B content of fresh and frozen peas from the same crop but not with the peas from different crops. The lesser amount of vitamin B in the frozen peas of the 1933 as compared with the 1934 crop cannot be attributed to prolonged storage since when tested they had been stored only 2 to 4 months.

In the studies of the effect of sprouting, in which ripened pea seeds were used as the basis of comparison, a daily dosage of 0.13 gm. of ripened pea seed was chosen as it represented approximately one-half of a fresh seed. An attempt was

TABLE 2

A comparison of the vitamin B content of sprouted and unsprouted pea seeds

MATERIAL	AMOUNT FED DAILY	NUMBER OF SEEDS REPRESENTED	NUMBER OF RATS	GAIN PER WEEK IN 4 WEEKS
	gm.			*gm.*
Pea seed	0.13	0.5	12	4.2
Sprouts and roots	0.09	1.0	8	5.7
Seed residue	0.05	1.0	8	—1.8

made to feed the dried sprouts and roots together in amounts corresponding to one-half of a seed but the doses were too small to furnish measurable amounts of vitamin B, and therefore at the end of 1 week of feeding the quantities were doubled and the tests continued for 4 weeks. The seed residues were also fed in amounts corresponding to one seed. Thus it was possible to compare the vitamin B values of the new plant substance (sprouts and roots) with the unsprouted pea seed, and also with the remnants of the germinated seed. The eight rats which received the dried sprouts and roots equivalent to approximately one seed (0.09 gm. per day), made an average gain of 5.7 gm. per week, whereas their litter mates receiving the corresponding residue, lost weight, indicating much destruction of vitamin B during the 14-day period of sprouting the seed. The results are shown in table 2.

The Lima beans were tested under three conditions, fresh, frozen and mature. When the fresh beans were fed in amounts of 0.7 and 1.0 gm. daily the average gains per week were 3.9 and 7.0 gm., respectively. Three rats each fed 1 gm. daily of the frozen Lima beans of the 1934 crop lost 6.3 gm. in 4 weeks. The mature seeds, some of which were used to grow the fresh beans, were fed in amounts equivalent on a dry weight basis to the daily dosage of fresh beans, with an average gain of 3.4 gm. per week for the period.

Since the fresh and frozen Lima beans were from two different crops, grown in two different localities, no comparison is possible with regard to the influence of freezing, but judging from the studies on the peas, the low vitamin B content of the frozen beans tested is in all probability due to climatic factors influencing growth and not to the freezing process.

The mature dried beans were less rich in vitamin B than the fresh seeds. It is not possible, however, to tell whether this is due entirely to maturity or partly to drying and storage, since the seed itself was grown in another locality and in the previous season. The experience with peas would indicate that maturity was the primary cause.

VITAMIN G EXPERIMENTS

The method used for the vitamin G studies was that of Bourquin and Sherman ('31) with an experimental period of 4 weeks. At the end of the first week of the depletion period, the rats were harnessed according to the anticoprophagy technic of Page ('32). Frozen peas of the 1933 crop were fed in amounts of 0.75, 1.0 and 1.5 gm. daily with resulting average gains of 1.7, 3.2 and 5.2 gm. per week, respectively. When the fresh peas of the 1934 crop became available, one group of ten rats was fed 1 gm. daily of the raw peas with an average gain of 4.4 gm. per week for the period, and another group of seven rats consuming the same peas cooked made an average gain of 4.2 gm. per week. A comparison of these results in figure 2 shows that there was

little difference in the vitamin G content of fresh, frozen and cooked peas of the same crop. There was, however, the same kind of difference between the vitamin G content of the frozen peas of the 1933 and 1934 crops as was shown in the vitamin B studies, the 1934 crop being richer in each case.

The mature pea seed (1933 crop) fed to a group of thirteen rats in doses of 0.27 gm. daily, equivalent to 1 gm. of fresh peas on a dry weight basis or one seed, resulted in an average gain of 3.4 gm. per week, as compared with 4.4 gm. on fresh peas.

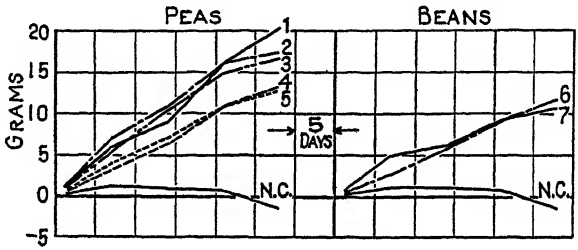

Fig. 2 Growth of animals receiving a vitamin G deficient diet plus daily supplements of the following: 1, 1.0 gm. frozen peas (1934); 2, 1.0 gm. fresh peas (1934); 3, 1.0 gm. fresh peas cooked; 4, 0.27 gm. pea seed equivalent to 1.0 gm. fresh peas; 5, 1.0 gm. frozen peas (1933); 6, 1.0 gm. fresh Lima beans; 7, 0.31 gm. Lima bean seed equivalent to 1.0 gm. fresh Lima beans; N.C., negative controls.

The sprouted pea seedlings were used in the way previously described for the vitamin B studies. For comparison mature pea seed was fed to three groups of rats on the basis of three-fourths, one, and one and one-half seeds daily producing average gains of 2.8, 3.5 and 5.1 gm. per week, respectively. To measure the amount of vitamin G in the sprouts and roots combined, the equivalent of one and one-half seeds a day was chosen. This amount of sprouts and roots produced an average gain of 4.9 gm. per week. The seed residue from one and one-half sprouted peas was also tested with the resulting gain of 2.9 gm. per week. The newly formed plant material proved to be as good a source of vitamin G as the original seed and the seed remnant still contained an appreciable amount of the vitamin.

The fresh Lima beans were fed in a dosage of 1 gm. daily and produced an average gain in ten rats of 3.9 gm. per week. When mature Lima bean seeds were fed on an equivalent weight basis, with 0.31 gm. of the dry seed as the daily dosage, there was an average gain of 2.7 gm. per week. Therefore there was practically no difference in potency between the fresh green beans and the mature seed. Table 3 summarizes the data for these tests and figure 2 shows the growth curves.

TABLE 3

Growth response of animals used to determine the vitamin G content of peas and Lima beans

MATERIAL TESTED	AMOUNT FED DAILY	DRY WEIGHT EQUIVALENT	NUMBER OF RATS	AVERAGE GAIN IN 4 WEEKS	AVERAGE GAIN PER WEEK
	gm.	*gm.*		*gm.*	*gm.*
Frozen peas (1933)	0.75	0.18	5	6.8	1.7
	1.00	0.25	5	12.8	3.2
	1.50	0.37	2	21.0	5.2
Frozen peas (1934)	1.00	0.25	7	20.4	5.1
Fresh peas (1934)	1.00	0.25	10	17.5	4.4
Cooked peas (1934)	1.00	0.25	7	16.9	4.2
Pea seed (1933)	0.27	0.25	13	13.4	3.4
Pea seed (1.5 seeds daily)	0.43	....	3	20.3	5.1
Sprouts and roots of 1.5 pea seed	0.14	....	10	19.5	4.9
Seed residue of 1.5 pea seed	0.11	....	8	11.8	2.9
Fresh Lima beans	1.00	0.29	10	11.8	3.0
Lima bean seed (1933)	0.31	....	8	10.9	2.7

Both fresh peas and fresh Lima beans proved to be fairly good sources of vitamin G, containing approximately 1 Sherman-Bourquin unit per gram. They were not as rich in vitamin G, however, as in vitamin B, thus furnishing additional evidence that seeds generally are relatively richer in vitamin B than in vitamin G. Fully ripened pea and Lima bean seeds were found to be about as rich in vitamin G as the fresh green ones, but further investigation of this point is desirable since the samples used were grown in different localities and under different conditions.

When peas were sprouted and allowed to grow for 2 weeks, there was a larger amount of vitamin G in the seedling, 3 to 6 inches high, than in the original seed.

SUMMARY

The vitamin B and G content of fresh peas has been determined for raw, cooked and frozen material and for mature seeds dry and sprouted; also for fresh Lima beans, both raw and frozen and for the mature seeds. Fresh raw peas were found to be a rich source of vitamin B, containing approximately 3 Sherman-Chase units per gram. Peas of the same season showed no loss due to freezing but a 26% loss in cooking 15 minutes. Peas of two seasons differed by almost 100%. Fresh Lima beans were only half as potent in vitamin B as fresh peas. Maturity in both peas and Lima beans resulted in loss of approximately half of the vitamin B.

Seedling peas grown on pure sand for 14 days showed loss of over half of the vitamin B but synthesis of vitamin G, the newly formed plant material being as good a source as the original seed, and the seed residue still containing a considerable amount of the vitamin.

Differences between fresh and frozen Lima beans of the same variety and season but grown in different regions show that locality must be taken into account in vitamin studies. Seasonal differences in vitamin G appeared in the frozen peas of the two crops studied just as in case of vitamin B.

The vitamin G content of both fresh raw peas and Lima beans was the same, 1 Sherman-Bourquin unit per gram. There was no loss of vitamin G in either in cooking or freezing.

In mature seeds of both kinds there was little loss of vitamin G as compared with the fresh seed.

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THE EFFECT OF THE ACID-BASE CONTENT OF THE DIET UPON THE PRODUCTION AND CURE OF RICKETS WITH SPECIAL REFERENCE TO CITRATES

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ONE FIGURE

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The question of the acid-base factor in the etiology and pathogenesis of rickets is one that has long excited interest. The experimental results have, however, been conflicting and the controversial discussions have failed to clarify the situation. Summaries of the earlier work have been given by Shelling ('25), Hess ('29), György ('29) and Goldblatt ('31) and in our earlier papers (Shohl et al., '28, Shohl et al., '32).

That rickets is associated with an acid metabolism and tetany with an alkaline, has been strongly advocated by Freudenberg and György ('22). The role of acidosis in the pathogenesis of rickets has recently been reviewed by Morris, Ford and Graham ('36) who could find no evidence that acidosis was "either a causal or associated factor in infantile rickets." The acid-base equilibrium of the blood shows little or no variation from the normal in either clinical rickets or infantile tetany, or, except in special cases, in experimental rickets (Shohl et al., '31). Therefore, efforts to show changes in metabolism have been directed toward studies of the excretion of acid and ammonia in the urine.

With regard to the effect of ingestion of acid our previous experiments have tended to confirm György's thesis, with

the reservation that, in the absence of vitamin D, the acid-base factor is secondary to alteration of the calcium-phosphorus intake. Obviously the acid-base content of the diet cannot be important clinically because the diet of infants, milk, is alkaline. Further, the acid-base effects could be shown experimentally in border line diets only. Third, it was evident that the acid-base effects were not direct but were complicated by the separate effects upon absorption and metabolism.

This last conclusion was based upon the following observations and reasoning. In rachitic tetany acid tends toward amelioration and alkali intensifies the condition. However, when acid, neutral and alkaline phosphates were given to rachitic rats, not the alkaline diets but the neutral ones produced the most severe tetany. From this we concluded that the total effect must be the resultant of two processes: 1) acid increased the absorption of Ca and P; alkali prevented it, 2) in metabolism after absorption, acid removed Ca and P from the body and alkali favored their deposition in the bones.

Hamilton and Schwartz ('33) have extended this thesis by a clever method of separating the two factors. They added $(NH_4)_2CO_3$ and NH_4Cl to 'normal' diets which were thus rendered alkaline in the intestinal tract and acid in metabolism. This alteration sufficed to make the diets rickets producing. It follows that a reversal of the factors should cause an opposite physiological effect. Thus, with diets which were acid in reaction but had an alkaline ash, namely with the addition of tartaric acid and sodium tartrate to diets which otherwise caused rickets, no rickets resulted. Their results are shortly to be published in detail.

Because the tartrates are too toxic to be used clinically it seemed desirable to extend this study to other organic acids. Further, we have recently shown (Brown et al., '32; Querido, '35; Shohl, '36) that when both the absolute amounts and the ratios of Ca and P are considered, rickets can be produced with many types of diets other than the classical high calcium-low phosphorus diets. Therefore, it was necessary to investigate whether the same acid-base effects would be obtained

in conjunction with different types of diet. It should then be possible to determine whether the acid-base alternations alone can be responsible for the etiology and also the cure of rickets in rats.

PLAN OF EXPERIMENT

Young albino rats were reared in our laboratory from Wistar Institute stock. The mothers were fed Sherman diet B (66% entire wheat flour, 33% dried whole milk, 1.3% NaCl) throughout pregnancy and lactation. The young were weaned to the same diet at 21 days. At 28 days of age, when weighing 45 to 55 gm., they were changed to the experimental diets and kept in groups of three in wide meshed cages without any bedding. They were fed these diets and distilled water. They were weighed weekly. After 21 days they were x-rayed, bled under light ether anesthesia, and autopsied. The bones of one hind leg were preserved for histological examination and those of the other carefully dissected, weighed, and the femur used for ash determination of the alcohol-ether extracted dry bones. The Ca and P of the serum were determined by the methods of Fiske and Logan ('31), and Fiske and Subbarow ('25), respectively. The individual x-rays were read and at the end of the experiment all the roentgenograms were compared and the original diagnoses checked and verified. The histological examinations and diagnoses therefrom were made without knowledge of the diets employed or the x-ray findings, through the kind cooperation of Dr. S. B. Wolbach.

As in our previous experiments, the two methods of diagnosis were in such close agreement that the results will not be differentiated in the following report.

The experimental diets consisted of 80% ground corn, 20% gluten flour and 1% NaCl, with additions of $CaCO_3$ and KH_2PO_4 to give the desired levels and ratios of Ca and P, as has been fully described in our previous paper (Shohl, '36). Diets containing any of these ratios were rickets producing provided the absolute amounts of Ca and P were low,

but were non-rachitogenic if the levels were high. The border line between rachitogenic and non-rachitogenic diets is shown in figure 1. The salt additions were made equivalent by computing them in terms of cubic centimeters of normal solutions per 100 gm. of diet, and were added as dry salts to the dry diet, and fed ad libitum. In a few cases the food intakes of individual rats were measured. These did not vary from those usually observed on similar diets so the procedure was discontinued. 1) Rickets production. $(NH_4)_2CO_3$ and NH_4Cl

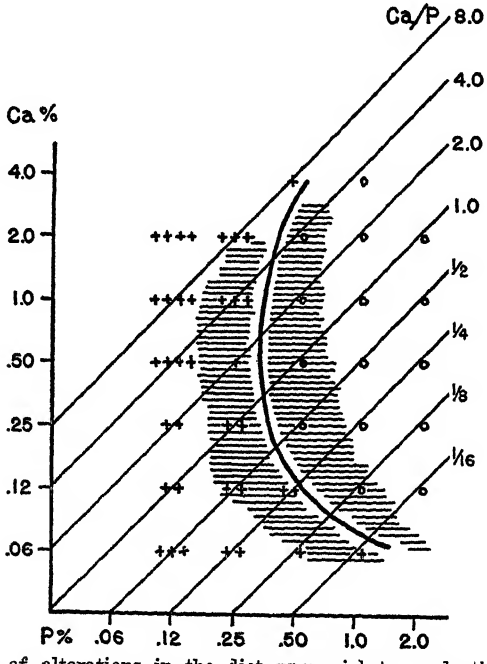

Fig. 1 Effect of alterations in the diet upon rickets production and rickets prevention. The abscissa and ordinate are laid off logarithmically to represent the diet percentages of P and Ca, respectively, and each point chosen is double that of the previous point. The curve represents the dividing line between rachitogenic diets on the left and non-rachitogenic diets on the right as described in our previous paper (Shohl, '36). The shaded area to the left of the line shows the extent of the field of successful rickets prevention with the use of citric acid-sodium citrate mixtures, or intensification of the degree of rickets by $(NH_4)_2CO_3$–NH_4Cl mixtures. The shaded area to the right of the line represents that in which non-rachitogenic diets were converted to rachitogenic diets by the use of $(NH_4)_2CO_3$–NH_4Cl mixtures, as described in the text.

were added to the diets. To test the effect of the component salts they were added separately and in combination in varying amounts to diet L ($Ca = 0.5\%$, $P = 0.5\%$) which is non-rachitogenic. The most effective combination was added to the diets either side of the border line in each of the eight ratio zones as shown in figure 1. 2) Rickets prevention. A citric-acid-sodium citrate mixture was added to the rachitogenic diet, St ($Ca = 1.0\%$, $P = 0.25\%$), separately and in combination. The mixture which was most effective was added to each of the border line rachitogenic diets. When the effects of these diets had been determined other organic acids—lactic, acetic, succinic, malic, malonic and tartaric were tested, using only one basal diet, that which most closely approached the classical diet no. 2965 of Steenbock and Black (diet St).

RESULTS

1. Rickets production. $(NH_4)_2CO_3$ and NH_4Cl were added separately and in combination to diet L as shown in table 1. In making the diet rachitogenic it is evident that the combination of the salts was more effective than either alone. Of the two the NH_4Cl is the more important for the $(NH_4)_2CO_3$ alone had no effect, but the acid-producing salt alone, if given in sufficient amount, made the diet rachitogenic.

A combination of 600 cc. 0.1 N $(NH_4)_2CO_3$ and 600 cc. 0.1 N NH_4Cl was added to rachitogenic and non-rachitogenic border line diets indicated in table 2. Most of the data represent six animals on each diet, run in groups of three so as to afford independent checks. Where the diet originally was rickets producing, the acid intensified the effect in every case, as evidenced by x-ray and histological examination. Except in the case of diet Y, the bone ash percentages were the same or lower than those of the rats fed the basal diets. Where the diet was one which produced normal bones at or near the border line (i.e., containing double the amounts of Ca and P per given ratio necessary to produce rickets), in every case the addition of $(NH_4)_2CO_3 + NH_4Cl$ made the diet rachitogenic. Where the diet was two stages from the border line

(i.e., containing four times the amounts of Ca and P), as in diets Q and O, the x-rays did not show rickets. However, the bones were thinner and the ash lower. They were considered border line cases. In the case of diet Q, with the addition of three times as much acid a definitely osteoporotic bone was produced and the per cent of ash was further lowered without producing definite rickets.

The growth of all the experimental animals, in accordance with our previous experience, was less than normal and varied from no weight increase to 12 gm. for the 21 days. It was impossible to correlate the growth with the presence or absence of rickets.

TABLE 1

Effect of additions of $(NH_4)_2CO_3$ and NH_4Cl to a non-rachitogenic diet [1]

ADDITIONS TO DIET L [1]		BLOOD SERUM		RICKETS [2]
NH_4Cl	$(NH_4)_2CO_3$	Ca	P	
cc. 0.1N per 100 gm. of diet	*cc. 0.1N per 100 gm. of diet*	*mg.%*	*mg.%*	
0	0	9.0	6.0	—
600	600	9.7	7.0	++
0	1200	9.7	7.6	—
600	0	6.6	6.0	+
1200	0	5.0	8.0	++

[1] Diet L which was used is described in text and in table 2.

[2] Histologic and x-ray diagnoses. The number of + signs indicates the severity of rickets; — indicates the absence of rickets.

From the data given in table 1 it is obvious that blood serum values give no clue as to the presence or absence of rickets. However, when the acid-producing salt was the sole addition the calcium was reduced and this effect was intensified when the amount of acid was increased. Morgan ('34) observed low calcium with acid diets.

The conclusion follows: acid-producing salts intensify rickets and have a definite but limited effect on the production of rickets.

TABLE 2

Effect of $(NH_4)_2CO_3$–NH_4Cl and citric acid-sodium citrate mixtures on the production of rickets and the amount of bone ash

	COMPOSITION OF BASAL DIET				RICKETS [1]			BONE ASH [2]		
						Basal diet + additions [4]			Basal diet + additions [4]	
	Ca/P	Ca	P	Acid-base [3]	Basal diet	$(NH_4)_2CO_3$ + NH_4Cl	Citric acid + sodium citrate	Basal diet	$(NH_4)_2CO_3$ + NH_4Cl	Citric acid + sodium citrate
		%	%	*cc. 0.1N*				%	%	%
Y	8/1	4.00	0.5	— 1865	±	+	—	46	51	50
St	4/1	1.00	0.25	— 430	+++	++++	—	39	39	49
W	4/1	2.00	0.50	— 865	—	+		50	47	
S	2/1	0.50	0.25	— 130	+	+	—	46	47	42
X	2/1	1.00	0.50	— 365	—	+		57	46	
K	1/1	0.25	0.25	— 30	±	+	—	46	41	37
L	1/1	0.50	0.50	— 65	—	+	—	48	42	
Q	1/1	1.00	1.00	— 195	—	—		50	46	
F	1/2	0.12	0.25	+ 40	+	++	—	41	36	42
G	1/2	0.25	0.50	+ 45	—	++		47	42	
O	1/2	0.50	1.00	+ 85	—	—		46	43	
B	1/4	0.12	0.50	+ 105	+	++	—	36	34	42
I	1/4	0.25	1.00	+ 195	—	+		41	41	
C	1/8	0.06	0.50	+ 135	+		—	37		36
D	1/8	0.12	1.00	+ 255	+	++	—	35	34	37
E	1/16	0.06	1.00	+ 285	+	++	—	32	33	38

[1] Histologic and x-ray diagnoses. The number of + signs indicates the severity of rickets; — indicates the absence of rickets.

[2] Determinations made on dried, fat-free femurs.

[3] Calculated per 100 gm. of diet. + = acid; — = base.

[4] Additions were: 600 cc. 0.1N $(NH_4)_2CO_3$ + 600 cc. 0.1N NH_4Cl per 100 gm. of diet, or 900 cc. 0.1N citric acid + 600 cc. 0.1N sodium citrate per 100 gm. of diet.

2. Rickets prevention. To analyze the action of citrates, variable amounts of sodium citrate and citric acid were added to diet St (Ca = 1.0%, P = 0.25%) separately and in combination (table 3). The remarkable effect of citrates in the prevention of rickets was not due to the citrate ion alone, for neither citric acid nor sodium citrate alone gave protection, either in the amounts used in combination with the

TABLE 3

Effect of additions of citric acid and sodium citrate to a rachitogenic diet[1]

ADDITIONS TO DIET ST[1]				RICKETS[2]
Calcium citrate[3]	Citric acid	Sodium citrate	Total	
	cc. 0.1N per 100 gm. of diet			
	0	0	0	+++
	300	300	600	+++
	0	600	600	++
	500	400	900	++±
	300	600	900	++
	900	0	900	++±
	1500	0	1500	++
	0	1500	1500	++
	900	600	1500	—
500	0	0	500	++±
500	0	400	900	++±
500	400	0	900	++
500	600	400	1500	+
500	400	600	1500	—

[1] Diet St which was used is described in the text and in table 2.

[2] Histologic and x-ray diagnoses. The number of + signs indicates the severity of rickets; — indicates the absence of rickets.

[3] Calcium citrate, where used, replaced an equivalent amount of calcium carbonate.

other, or when the total amount of citrate was given in either form.

The citrate combination which had been found most effective, 900 cc. 0.1 N citric acid and 600 cc. 0.1 N sodium citrate, was added to each of the rachitogenic diets close to the border line. Whether high alcium-low phosphorus, low calcium-high phosphorus, or low calcium-low phosphorus, the

diets which had previously produced rickets now caused no rachitic lesions, as evidenced by x-ray and histologic studies (table 2). The bone ash showed an increase in six cases and a diminution in two cases. No effort was made to explore the extent of the field of this salt effect, but the diets studied included the classical rickets producing diet. To attain lower phosphorus diets than those used it would have been necessary to diminish the P more than could be done with the use of corn, and such alterations of the diet would involve P starvation and other problems lying outside the field of this investigation.

The blood serum Ca and P showed no alteration from that of classical low P rickets when the citrate mixtures did not prevent rickets. With intermediate and effective amounts the P tended to rise irregularly and the Ca to be depressed.

The $CaCO_3$ present in diet St causes an excess of 430 cc. 0.1 N alkali per 100 gm. of diet. It was desired to test whether the neutralization of the carbonate outside the body was the same as that inside the body. Therefore, a diet was made with an equivalent amount of calcium citrate instead of the carbonate. Various additions of citric acid and sodium citrate were made to this diet as shown in table 3. In no case was the diet effective in preventing rickets until the original values (900 cc. citric acid and 600 cc. sodium citrate) were reached. Neither the acid nor the alkali alone was sufficient to bring about the prevention of rickets; both citric acid and sodium citrate were necessary. One must conclude that the factors of acid in absorption and alkali in metabolism cooperate in the total effect.

As a further test the curative action of the citrates was investigated. Rickets was produced by feeding animals diet St for 21 days. They were then fed, in addition, the citric acid-sodium citrate mixture shown above to be effective in the prevention of rickets. As early as 7 days following this addition to the diet, healing was evidenced by x-ray, serum analysis and ash determination.

If the results obtained with citrates were due solely to the acid-base effect on metabolism, it follows that similar effects should be produced with other organic acids and their salts. Accordingly a test was made with diet St using equivalent amounts of tartaric acid and sodium tartrate. The diet was rendered non-rachitogenic by these additions, as shown in table 4. This confirms the preliminary report of Hamilton, referred to above. However, when other acids, acetic, lactic, malic, malonic and succinic acids, and their sodium salts were used, the diet still produced rickets, as evidenced by both x-ray and histologic examination, and the blood serum showed the findings characteristic of low phosphorus rickets. Although the acid-base content of the diet is important, for, only in the presence of the acid and its salt are the citrates effective in rickets prevention, the results obtained with citrates and tartrates must be due to their specific properties and not to their acid-base effect alone.

TABLE 4

Effect of additions of various organic acids and their salts to a rachitogenic diet[1]

ADDITIONS TO DIET ST[1]	RICKETS[2]	BONE ASH[3]
cc. 0.1N per 100 gm. of diet		%
Diet St	+++	34
900 citric acid + 600 sodium citrate	—	49
900 tartaric acid + 600 sodium tartrate	—	38
Lactate[4]	+++	31
900 lactic acid + 600 sodium lactate	+++	35
Lactate[4] + 900 lactic acid + 600 sodium lactate	++	40
900 acetic acid + 600 sodium acetate	+++	36
900 malic acid + 600 sodium malate	+++	
900 malonic acid + 600 sodium malonate	+++	
900 succinic acid + 600 sodium succinate	+++	

[1] Diet St which was used is described in the text and in table 2.

[2] Histologic and x-ray diagnoses. The number of + signs indicates the severity of rickets; — indicates the absence of rickets.

[3] Determinations made on the dried, fat-free femurs.

[4] In these cases the $CaCO_3$ of diet St was replaced by an equivalent amount of calcium lactate.

DISCUSSION

Schloss ('17) objected to the acid theory of rickets, on the basis that acid ingestion leads to osteoporosis and not to rickets, and second that alkali administration does not heal rickets. For experimental rickets these objections are no longer valid.

From the data presented it is clear that the acid-base content of the diet is important in the etiology of experimental rickets in rats. In our previous experiments (Shohl et al., '32) the diets chosen for study were so near the border line that they should not strictly be classed as either rachitogenic or non-rachitogenic. With neutral diets a mild healing type of the condition was present. Under these conditions acid diets intensified rickets and alkaline diets prevented or cured it. Similarly Morgan ('34) showed that acid diets intensified the degree of rickets in dogs. However, the exact effect of alkali is not clear from her data. In any event the basal diet apparently was not only rachitogenic, but sufficiently so that with the amount of cod liver oil she used it was not clear that healing took place. So far as we are aware, we produce here the first clear evidence that changes in acid-base alone can convert a non-rickets producing diet into a rickets producing diet or vice versa. Moreover the result is definite, not for a single diet but for all types of diet in which calcium and phosphorus are the main variables.

That the addition of NH_4Cl alone produces rickets indicates that the Hamilton thesis, that alkaline reaction in the intestinal tract is a necessary factor, has not been confirmed. That $(NH_4)_2CO_3$ enhances the effect is, however, evidence that the alkaline reaction is a supporting factor.

It is further clear that the combination of acid reaction with alkaline ash is not alone responsible for rickets prevention in diets which are rachitogenic to this degree because it is effective only when tartrates or citrates are used. From our study of the citrates, however, it is shown that neither an acid reaction, nor an alkaline ash alone is sufficient and that both are necessary to prevent rickets in these diets. That

orange juice increased the retentions of Ca, P, Mg and N in children was shown by Chaney and Blunt ('25). The beneficial effect was attributed to the citric acid-alkaline citrate. The significance of these findings as applied to rickets was emphasized by Querido ('35).

Morris and MacRae ('32) gave NH_4Cl to infants with healing (?) rickets plus tetany and reported that calcification was not prevented. Acid milk has been used to cure rickets, but unless the acid is in too great an excess this should simulate the conditions of increased acid reaction in the ingested food and a mineral residue which is still alkaline. However, Hess and Matzner ('24) stated that acid milk did not cure rickets. Zucker's ('22) original experiments showed that acid added to the diet rendered it less rickets producing. However, he informed the author personally that unpublished data showed that when the acid was increased beyond the neutral point the degree of rickets was then again increased.

In the present study, the diets used varied in acidity as shown in table 2. Those with the most $CaCO_3$ were most alkaline and those with KH_2PO_4 were most acid. It can be argued that the experimental diets should all be neutral. Such a plan was devised by the use of acid and alkaline phosphate mixtures, and of $CaCO_3$-$CaCl_2$ mixtures. The reasons why such diets were not used were discussed in the previous paper (Shohl, '36). Such a regime might give a different curve for the border line of rickets production, but a new set of variables thus introduced would prevent comparison with our previous results. However, our present conclusions would not be altered, but could only be more exactly defined because the effect was shown with acid and alkaline as well as neutral diets.

From the per cent ash one cannot differentiate between osteoporosis and rickets; the value becomes a comparative measure only when the lesions are known to be similar—i.e., by histologic examination. This is obvious from inspection of the data on the animals fed lactates and tartrates, shown in table 3. The bones of the former showed a greater per cent

of ash than the latter, but the animals receiving lactates had rickets and those receiving tartrates had none.

Because no light has been thrown on the mechanism of rickets production or prevention by either growth or blood studies it seems that metabolic studies are necessary to determine how these results are brought about, and how the metabolism of Ca and P are altered. In light of the well-known property of citrate solutions to form complex calcium ions, as demonstrated by Hastings et al. ('34) an attractive field for further investigation is suggested.

By the addition of salt mixtures to make diets acid in reaction and alkaline in ash or vice versa the acid-base effects of diets on the production and prevention of rickets have been brought out more clearly than ever before.

Alterations in the Ca and P intake have previously been shown to cure experimental rickets. The addition of citrates, either alone or in conjunction with Ca and P additions without vitamin D or ultraviolet light, offers a second method for possible therapeutic application to infantile rickets.

SUMMARY AND CONCLUSIONS

The diets used were made to contain eight different ratios of calcium to phosphorus. In each of these ratios there are zones where rickets were produced when the absolute amounts were low and no rickets when the amounts were increased.

1. a. The addition of NH_4Cl–$(NH_4)_2CO_3$ mixtures to nonrachitogenic diets, in each of the eight zones, renders them rachitogenic. Further, the same additions to the rachitogenic diets in each of the eight zones, intensifies the severity of the rickets produced.

b. The NH_4Cl is the more important moiety, though the $(NH_4)_2CO_3$ enhances the effect.

2. a. The addition of citric acid-sodium citrate mixtures to rachitogenic diets in each of the eight zones alters them so that they no longer produce rickets.

b. Both the acid reaction and the alkali ash factors are necessary to accomplish this result.

c. In the prevention of rickets, the citrates and tartrates alone, among the organic acids tested, were effective; therefore their action cannot depend entirely upon their acid-base effects.

It is a pleasure to acknowledge the assistance of Dr. S. B. Wolbach for the histological interpretation of the bones, of Miss E. MacLachlan for the Ca and P determinations and of Dr. E. Vogt for the x-ray pictures and assistance in their interpretation.

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SYMPTOMATOLOGY AND PATHOLOGY OF POTASSIUM AND MAGNESIUM DEFICIENCIES IN THE RAT

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TWO PLATES (EIGHT FIGURES)

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Although it has long been recognized that potassium and magnesium are essential for animal and plant life, the effect of a deficiency of these elements on animals has been studied only recently. Information concerning the histopathology of potassium or magnesium deficiency is especially scanty.

Very little work has been done on the symptomatology of potassium deficiency. Miller ('23) reported a very slow growth and an abnormal alertness as the two principal symptoms of rats fed a synthetic diet low in potassium. Leulier and Vanhems ('34) found that young rats failed to grow and died in 12 to 24 days when fed a diet deficient only in potassium. We were unable to find any reports on the histopathology of potassium deficiency.

The symptomatology of magnesium deficiency has received considerable attention in recent years, probably due largely to the excellent reports of McCollum and Orent ('31) and of Kruse, Orent and McCollum ('32 a) on the rat, and of Orent, Kruse and McCollum ('32) on the dog. Leroy ('26) had earlier reported that mice died in 24 to 35 days, when fed a low magnesium diet, but mentioned no other symptoms. In the main the findings of the above workers on the symptomatology in magnesium deficiency of the rat have been confirmed by Brookfield ('34). Greenberg and Tufts ('35 a, '35 b)

produced only the symptoms of convulsions in rats on a low magnesium diet. Moreover, the convulsive seizure was not spontaneous, but had to be induced by a suitable stimulus, and could not be induced if the animals received sufficient vitamin G. They also found that the time required for the appearance of convulsions was dependent on the vitamin G intake. Cramer ('32) noticed no effect on the health of rats on a low magnesium diet, during a 6-week experimental period.

Recent work indicates that, under certain conditions, symptoms of magnesium deficiency may develop in livestock. A peculiar disease called 'grass tetany' is known to occur in dairy cows. The etiology of the disease is obscure, but it has been shown (Sjollema, '32 a, '32 b, and Sjollema and Seekles, '32) that a very low blood magnesium is present. The symptoms of the disease strongly resemble those of magnesium deficiency and the above workers and also Pulles ('31) found that injections of a mixture of $CaCl_2$ and $MgCl_2$, if done in time, would cure the disease. Following the reports by Kruse, Orent and McCollum ('32a), Sjollema ('32 c) reached the conclusion that 'grass tetany' is primarily caused by a low magnesium level in the blood. Green, Allcroft and Montgomerie ('35) reported a low blood magnesium in equine transit tetany and considered this disease to be similar to 'grass tetany' in cows. Calves, which have been reared mainly on a milk diet, may develop magnesium deficiency, as evidenced by a low plasma magnesium and tetany (Duncan, Huffman and Robinson, '35). Huffman and Duncan ('36) later found that both the low plasma magnesium and the tetany in calves were prevented by feeding magnesium in the form of the oxide, the carbonate or natural foods.

Although it is highly improbable that the average diet of humans is low in magnesium, apparently certain occasions may arise when the intake of this element is insufficient. Hirschfelder ('33 and '34) has reported a low blood magnesium in ten patients, which was accompanied by recognizable symptoms of magnesium deficiency; these patients were receiving special soft diets, which included milk; oral administration of $MgSO_4$ to three of the patients resulted in a

rise in blood magnesium and a disappearance of convulsions. Daniels and Everson ('36) conducted mineral metabolism experiments on pre-school children who received complete diets. It would appear possible, from their analytical data on urinary magnesium, that some of the children may have been on a previous dietary of sub-optimum magnesium content.

The pathology of magnesium deficiency has received some attention. Kidney lesions were found by Cramer ('32) in rats fed a low magnesium diet. Brookfield ('34) reported degenerative changes in the liver as well as in the kidney of the rat. Moore, Sholl and Hallman ('36) recently reported the pathology associated with a low blood magnesium in dairy calves. They found that "in general the microscopic picture involved a basophylic hyaline-like necrosis of the collagenous and yellow elastic connective tissue elements of the heart, blood vessels, spleen, peritoneal and pleural surfaces of the diaphragm." Chemical changes in the blood (Kruse, Orent and McCollum, '32 b and '33), in the bone (Orent, Kruse and McCollum, '34), and in the mineral metabolism (Kruse, Schmidt and McCollum, '34) have also been reported.

During a study of vitamin G deficiency in the rat and the chick in this laboratory, diet 231 of Keenan, Kline, Elvehjem and Hart ('35) was used; atypical symptoms were encountered when the yellow corn, wheat middlings, and casein were extracted with dilute acetic acid instead of being heated. These symptoms were traced to a deficiency in the diet of both potassium and magnesium. Interesting symptomatology and pathology were observed in rats in each instance. A search of the literature revealed scanty information concerning potassium deficiency, and contradictory findings in magnesium deficiency. Because of these facts, a somewhat detailed study of each deficiency has been made on the rat. The results are presented in the following pages.

EXPERIMENTAL PROCEDURE

The rats were started on experiment at an average weight of 55 gm. Their average age was 24 days, with a range of from 22 to 25 days. Individual metal cages were used in all experiments. All rats received the basal diet ad libitum; the food was weighed out daily. Distilled water was always available. Some groups received their respective basal diets only; others received the basal diet plus added vitamin B. The vitamin B supplement was a fuller's earth adsorbate of a brewer's yeast extract. Ten milligrams of adsorbate was given on alternate days. Pigeon tests showed the above quantity to contain 3 international units of vitamin B (Salmon and Goodman, '34).

The same basal diet was used for all the feeding experiments; it varied only in the inorganic supplements. These were added, when necessary, at the expense of the extracted grain mixture. Essentially this diet was the same as diet 231 which had been used by Keenan, Kline, Elvehjem and Hart ('35). It differed only in the treatment of the yellow corn, wheat middlings, and casein. Instead of heating these constituents they were subjected to extraction. The casein was extracted as described by Salmon and Goodman ('34). About 40 pounds of the grain mixture, consisting of 59 parts ground yellow corn and 25 parts wheat middlings, was extracted by percolation in a large monel metal kettle with 0.2% acetic acid for 6 days; tap water was used the first 5 days and distilled water the sixth day. Distilled water alone was used the seventh day. On the eighth day the extracted grain mixture was pressed as dry as possible in a filter press, and was placed in shallow enamel pans and dried in a forced draft oven at 60 to 70°C.; it was then ground to the desired degree of fineness. The diets were made up about every 10 days. When not in use they were stored in a cold room at a temperature of about 3°C.

The diets were fortified with salt mixture no. 186 (Guerrant and Salmon, '30), or with simple salts of C.P. grade. Magnesium, in the form of the trihydrated sulfate, and potassium, in the form of the chloride, were added in such amounts

as would furnish the same quantity of magnesium and potassium as were contained in 4% of salt mixture no. 186. The chloride content of the diets containing simple salts was maintained at a constant level. The composition of the basal diets is given in table 1.

Besides the rats on the potassium and the magnesium deficient diets (231E + Mg and 231E + K, respectively), a group of rats was fed a diet deficient in both potassium and magnesium (231E). This group served as a negative control.

TABLE 1

Percentage composition of basal diets

INGREDIENTS	231E	231E + MG	231E + K	231E + MG + K	232
Extracted grain mixture [1]	84.0	83.5	82.6	82.1	83.0
Extracted casein [2]	12.0	12.0	12.0	12.0	12.0
Cod liver oil	1.0	1.0	1.0	1.0	1.0
Salts 186 [3]	....	....	....	....	4.0
NaCl	1.0	1.0	....	....	....
$CaCO_3$	1.0	1.0	1.0	1.0	....
$Ca_3(PO_4)_2$	1.0	1.0	1.0	1.0	....
$MgSO_4 \cdot 3H_2O$	....	0.5[4]	....	0.5[4]	....
KCl	....	....	1.0[5]	1.0[5]	....
$NaHCO_3$	....	....	1.4[6]	1.4[6]	....

[1] Ground yellow corn 59 parts and wheat middlings 25 parts. This mixture was extracted with 0.2% acetic acid for 6 days and distilled water 1 day.

[2] J. Nutrition, '34, vol. 8, p. 1.

[3] J. Biol. Chem., '30, vol. 89, p. 199.

[4] This quantity of $MgSO_4 \cdot 3H_2O$ furnishes the same amount of Mg as is supplied by 4% of salts 186.

[5] This quantity of KCl furnishes the same amount of K as is supplied by 4% of salts 186.

[6] This quantity of $NaHCO_3$ furnishes the same amount of Na as is supplied by 1% of NaCl.

A positive control group received both potassium and magnesium in the form of the simple salts (diet 231E + Mg + K). As an additional positive control, a group of rats was fed the basal grain mixture supplemented with the complex salt mixture no. 186 (diet 232).

With the exception of a few cases in which tissues showing the various stages of the skin lesions found in animals on the

magnesium deficient diet were wanted for microscopic study, the animals on the deficient diets were allowed to continue on experiment until moribund. Control animals were killed at comparable periods. When necessary, the animals were killed with chloroform. Table 2 shows the number of animals on which necropsies were made, and also the number used for histopathological studies.

The tissues used for microscopic study represent a cross section of the groups on which gross observations were made. In the case of the groups on the magnesium deficient diet, only the tissues of animals known to have died in convulsive seizures were used. More time and more animals were not devoted to this phase of the work because within any given group the findings were remarkably consistent. The tissues were removed immediately after death, fixed in 10% formol-saline, embedded in Parlodion and sectioned at 15 μ. They were stained with Harris' hematoxylin and counterstained with either eosin Y or Triosin.[1]

RESULTS

Potassium deficiency

Growth and duration of life. The rats on the potassium deficient diet made practically no growth, and at death had lost an average of about 10% of the starting weight (table 2). The average time to death or closing out was 23 days. Vitamin B addition had no significant effect on weight or length of life.

Symptomatology. Early in the experiment, the rats began to show a lethargic condition which gradually progressed into coma and death. Accompanying the lethargic symptoms was a progressive degree of abdominal distention which was usually very marked by the time the animals became moribund. The skin was pale and somewhat cyanotic; the hair was short and fur-like. In a few cases, pendulous sacs were seen in the region of the thyroid gland and the manubrium of the sternum.

[1] Triosin is a differential plasma stain manufactured by Albert E. Galigher, Inc., 1228 Solano Avenue, Berkeley, California. In our experience, it has proved more selective than eosin.

Gross pathology. At necropsy, a tremendous amount of pathology was found, the abdominal cavity being the site of the greatest amount of change. Severe ascites was usually present, there often being as much as 4 to 5 cc. of clear straw-colored fluid. In a few cases, hydrothorax and hydropericardium were noted.

TABLE 2

Average growth and duration of life of rats on the K and Mg deficient diets, together with the number of rats used for gross and histopathological studies

BASAL DIET	SUPPLEMENT	GROWTH STUDIES				PATHOLOGY STUDIES	
		Number of rats	Maximum increase in weight	Final change in weight [1]	Time to death or closing out	Rats used for gross pathology	Rats used for histo-pathology
			gm.	*gm.*	*days*		
231E	None	12	0	−6	26	7	4
231E	Vitamin B [2]	10	0	−7	23	10	5
231E + Mg	None	11	0	−6	23	10	5
231E + Mg	Vitamin B [2]	18	1	−5	21	8	5
231E + K	None	25	11	5	34	16	10
231E + K	Vitamin B [2]	24	17	12	37	15	8
231E + Mg + K	None	6	47	47	56[3]	18[4]	4
231E + Mg + K	Vitamin B [2]	6	48	48	56[3]	18[5]	4
232	None	6	53	53	56[3]	6	..
232	Vitamin B [2]	5	49	49	56[3]	5	..

[1] The difference between the average starting weight and the final weight at death or closing out.

[2] Ten milligrams of a fuller's earth adsorbate of a brewer's yeast extract given on alternate days to each rat. Pigeon tests showed this quantity to contain 3 I.U. of vitamin B.

[3] No deaths occurred in any of these four groups.

[4] Six rats were killed at 21 days, six at 33 days, and six at 56 days. The former two groups of six served as controls for groups 231E + Mg and 231E + K, respectively.

[5] Six rats were killed at 21 days, six at 36 days, and six at 56 days. The former two groups of six served as controls for groups 231E + Mg + vitamin B and 231E + K + vitamin B, respectively.

The intestinal tract was tremendously enlarged, markedly atonic, and had a translucent agate-like appearance. The ileum and lower jejunum were the site of the severest changes but, in a few cases, the whole intestinal tract was affected. In the ileum, thickened annular areas were observed, usually

surrounding Peyer's patches; these thickenings gave the intestine a 'beaded' appearance. In such areas the congestion was often so marked that it was thought to be hemorrhage. Intervening portions of the intestinal tract appeared anemic and were often collapsed.

In the predominating number of animals, intussusceptions were found in the ileum and lower jejunum. As many as four were observed in a single animal. In one case, a large intussusception involving the major portion of the ileum had entered the cecal pouch. No gross evidence of infarction of the intestine was noted.

The mesenteric lymph nodes were markedly enlarged, translucent, and surrounded by opalescent jelly-like material. Accumulations of this material were also observed in the pendulous sacs in the region of the thyroid and also in the axilla. The kidneys were enlarged and very pale; when they were sectioned longitudinally, fluid was seen to exude from the cut surface. Commonly, there was a marked congestion of the suprarenal glands and, in one case, hemorrhage. The liver was somewhat pale and appeared flabby.

The pancreas was usually markedly dissociated into its constituent lobules which were loosely bound together by opalescent jelly-like material. Often the scattered pancreatic tissue had a distribution similar to that of the greater omentum.

On the lateral aspect of the right ventricle of the heart, at the apex and, less frequently, on the left ventricle were noted opaque areas of variable size.

Necropsy findings on animals on the potassium deficient diet supplemented with vitamin B differed from those not receiving this supplement in only one particular. In these animals, intussusceptions were found to be a much less frequent occurrence.

Microscopic pathology. The most consistent and most severe changes were found in the lower jejunum and ileum. However, in a few cases the stomach, duodenum and upper jejunum were the site of mild effects. Two distinct types of change were observed which corresponded in position to the

changes noted at necropsy. In areas corresponding to the enlarged, annular, or 'beaded' portions of the intestine, the Peyer's patches were usually markedly congested, edematous, rarefied and contained numerous mononuclear phagocytic cells. No hemorrhages were noted. The villi were seen as apparently confluent, distended bodies which contained innumerable lymphocytes and phagocytes (plate 1, B). The mucosa, particularly at the tips of the villi, was markedly edematous, vacuolated, and the nuclei of the columnar cells were usually pycnotic. Frequently the tip of the villus was the site of an accumulation of phagocytes in various stages of degeneration which formed a 'cap.' Sloughing of portions of the mucosa with apparent rupture of the villi which allowed its contained lymphocytes and phagocytes to enter the intestinal lumen was occasionally seen. Numerous phagocytic cells, both mononuclear and polymorphonuclear, were consistently found within the lumen of the intestine in this region. They were apparently embedded in the jelly-like material observed at necropsy. The borders of this type of lesion were sharply defined.

The second type of lesion corresponding in position to the tissue intervening between the 'beaded' portions showed more of an edematous change. The vascular elements were markedly congested. The submucosa as a whole was usually very rarefied, edematous, and distended, but frequently the distention was confined to the tips of the villi giving them a 'clubbed' appearance (plate 1, A, C). The mucosal epithelium was usually shortened and thickened, appeared atrophic, and the nuclei were pycnotic. However, the columnar cells at the tips of the villi were frequently edematous and vacuolated, and were 'capped' by accumulations of phagocytes. The lumen of the intestine in the region of this type of change contained few phagocytic cells.

The large intestine occasionally showed mild edema in the submucosa and congestion of blood vessels. The mesenteric lymph nodes were enlarged, edematous, rarefied and contained considerable numbers of mononuclear phagocytes.

In the portions of the intestines involved in intussusceptions, the same changes were observed as in other portions of the lower jejunum or ileum, but no evidence of infarction was noted.

Massive erosions involving both the endocardium and the myocardium were found in both ventricles of the heart. These lesions varied in size and had rather sharply defined borders. There was usually complete destruction of cardiac musculature and replacement with scar tissue and numerous phagocytes (plate 1, D). In several cases, the erosion had almost perforated the heart wall. There was usually a variable amount of interfibrillar infiltration of lymphocytes in the tissues in close proximity to the erosions. The cardiac vessels were usually engorged. In a few cases, whole muscle bands stained deeply, the nuclei appeared pycnotic, and the cells shrunken. Other muscle bands on either side, if not involved by the erosive type of lesion, were apparently normal. Occasionally, the erosions were noted in the valvular tissue, but no changes were ever found in the auricles.

Considerable engorgement of blood vessels and foaminess of hepatic cells were the only findings in the liver.

The pancreas was usually markedly dissociated into fragments of variable size embedded in the jelly-like mass. The interlobular connective tissue was practically absent. The sections stained poorly, being homogeneous and hazy in appearance. Acinar tissue appeared edematous. In a few cases, the number of islands of Langerhans seemed diminished and those remaining appeared somewhat edematous and hypertrophic. Numerous phagocytes were usually seen in the jelly-like material.

Diffuse tubular nephritis was present in all kidneys observed (plate 2, A). The epithelium of the tubules was markedly edematous, vacuolated, and had sloughed in some cases. Pycnosis of nuclei was common. There was a considerable degree of congestion of the renal blood vessels. Little change was seen in the glomeruli.

In the suprarenals, there was marked engorgement of blood vessels. The cortical cells stained poorly and, in a few cases, appeared rarefied and edematous.

The tissues of animals which had received the diet containing an adequate amount of potassium showed no microscopic changes.

Magnesium deficiency

Growth and duration of life. The rats on the magnesium deficient diet gained an average of about 10 gm., mostly in the first 3 weeks on experiment. Some of the gain in weight was lost by the time death occurred at an average time of 34 days (table 2).

Symptomatology. The animals began to show mild symptoms of hyperirritability as early as the end of the first week on experiment. This hyperirritability became more marked as the experiment continued.

About the end of the first week on experiment a generalized hyperemia of the skin was observed, which was succeeded about the tenth day by an erythema which was most severe on the dorsum of the back and on the flanks. In some cases, the erythematous change was observed in the scapular, cranial, masseteric, and mandibular regions, and at the roots of the ears. Although the erythema was occasionally generalized, it was most often present in discrete circular areas of variable size. Intervening skin was apparently normal. Occasionally, pronounced edema was seen in the pedal portion of the posterior extremities and in the nasal region.

Succeeding the erythema the greater number of animals developed more or less extensive purpural skin hemorrhages. They were confined to the areas which had shown erythema. Usually a variable amount of eschar formation followed the purpura. The eschars were both of the dry and moist type, and were found most consistently on the back in the sacral region and on the flanks. However, they were also observed in a few cases in the scapular, cranial, masseteric, and mandibular regions. They were occasionally seen as confluent masses of escharotic tissue but were more often discrete

and circumscribed (plate 2, B). Following the escharotic stage, exfoliation and healing took place leaving the skin mildly scaly and the area denuded. The duration of the escharotic stage was extremely variable, occasionally lasting throughout the remainder of the experiment. The purpural and escharotic changes were not necessarily a sequence to the erythema, but were never observed unless the animal had previously shown it.

Tonic-clonic spasms were observed as early as the seventeenth day, and as late as the fifty-first day. The average time of onset was 30 days. The symptomatology of these seizures was essentially the same as that described by Kruse, Orent and McCollum ('32 a) with the following exception: in only one animal was more than one seizure noted during an attack. This animal had three severe seizures in succession.

Death was characterized by its suddenness and its unpredictability. However, the fact that most animals were found in positions similar to those observed during convulsive seizures is suggestive that death followed such an experience.

Gross pathology. At necropsy, few pathological changes could be found in the gross. Occasionally, pigmentation and apparent scarring of the skin were observed in areas which had previously been affected by the purpural and escharotic lesions. The intestinal tract was mildly atonic, congested and, in a few cases, mild gaseous enteritis was seen. The suprarenal glands were usually congested. The heart appeared somewhat enlarged and flabby.

In the greater number of animals necropsied, the liver appeared somewhat pale, mottled, and flabby, but in nine animals massive lesions were observed. These changes were of two types. The first type, which was found in five animals, consisted of dark red areas in which the lobulation of the liver appeared as in mosaic. The borders of these lesions were discrete and circumscribed, and apparently had no particular site of distribution. In the second type, found in two animals, the livers were markedly contracted by extensive cicatrizing

lesions which sent out finger-like projections of scar tissue deep into the liver substance. They were apparently an older form of change than the mosaic type. In two cases, both the cicatrizing and the mosaic type of change were observed in the same liver.

Animals on this same diet supplemented with vitamin B showed no significant difference from those not receiving this supplement.

Microscopic pathology. Considering the violent nature of death in rats on this diet, very little pathological change was found when the microscopic examination of the tissues was made.

The intestinal tract evidenced only a mild congestion of the vascular elements. The heart was normal as was the pancreas. In the kidneys occasional tubules showed mild degenerative changes. There was no calcification. The suprarenals were markedly congested.

Sections of the skin in the active erythematous stage showed only marked engorgement of blood vessels and edema. In the escharotic stage, there was edema, mild infiltration of white blood cells, and at times sloughing of the cuticular layer. In sections of skin taken when all gross evidence of the lesion had disappeared, there was generalized sloughing of the cuticular layer and, in some cases, scarring.

In the greater number of livers examined, mild foaminess of the cellular elements and marked congestion of the vascular tissues were the only changes noted. However, in the livers which had shown the massive cicatrizing lesions at necropsy, the changes were diffuse and varied. Practically all liver cells were affected to some extent. Vascular proliferation and perivascular accumulations of small round cells were common. Numerous areas of scar tissue accompanied by marked infiltration of lymphocytes and phagocytes were seen (plate 2, C). In some places the tissues were practically acellular, and had a homogeneous hyaline-like appearance.

The second type of lesion in which, at necropsy, the lobulation of the liver was seen as if in mosaic, appeared to be a

recent involvement. No scarring was observed, but vascular proliferation and perivascular infiltration of small round cells were common. In most areas, the damage was confined to the periportal regions whereas in others it was diffuse (plate 2, D). The degenerated areas were practically acellular except for a few pycnotic nuclei and numerous phagocytes containing green-yellow ingested material. Occasionally, gigantic nuclei were observed which appeared normal in structure. The borders of the lesions were rather sharply defined, but all liver cells showed a marked foaminess of the cytoplasm.

The tissues of animals which had received the diet containing an adequate amount of magnesium showed no microscopic changes.

Magnesium and potassium deficiency

The rats on the diet which was deficient in both potassium and magnesium resembled the potassium deficient rats in so far as change in weight and time to death were concerned (table 2). They evidenced a considerable degree of hyperirritability in the early part of the experiment, which corresponded to that observed in animals on the magnesium deficient diet. In contrast to the animals on the magnesium deficient diet, however, the hyperirritability soon disappeared and the animals began to show more of the lethargic type of symptoms seen in animals on the potassium deficient diet.

No skin lesions or erythema were observed in these animals. They became progressively more lethargic and weakened; abdominal distention became marked and, at the time of death, they were usually in a comatose condition.

Necropsy and microscopic findings in this group were essentially the same as those observed in animals on the potassium deficient diet and will not be repeated here. The addition of vitamin B had no apparent effect.

Positive controls

No deaths occurred, either in those rats receiving the simple potassium and magnesium salts or in those receiving the complex salt mixture, during an 8-week experimental period. A

steady gain in weight was maintained, which was practically identical in both groups. The addition of vitamin B had no appreciable effect on the results obtained from either group. The data are summarized in table 2. Similarly, no gross pathological changes were observed in either group. Due to the lack of gross pathology, microscopic studies of tissues of rats which received the complex salt mixture were not made.

DISCUSSION

The important role that potassium plays in animal metabolism is dramatically illustrated by the short duration of life and the pronounced tissue changes occurring in young rats fed a diet deficient in this element. The duration of life and effect on growth obtained by Leulier and Vanhems ('34) are in substantial agreement with our data. They did not mention any other symptoms, however. Our rats developed short fur-like hair, cyanosis, abdominal distention, and lethargy leading to coma and death. It is possible that Miller's diet ('23) was not as low in potassium as the analyses indicated because his rats did not die during a 17-week experimental period. In contrast to the abnormal alertness of his rats, our animals exhibited a lethargic condition. His rats made a slow gain in weight; our rats lost weight slowly from the beginning of the experiment. The fact that the rats on our magnesium deficient diet showed an initial gain in weight is further proof of the extreme importance of potassium in animal nutrition.

Many of the pathological changes such as ascites, hydrothorax, hydropericardium, and the marked edematous effects observed in the intestines of the rats on the potassium deficient diet would tend to indicate a pronounced disturbance of fluid metabolism. Such a result might be expected from a lack of an element so intimately concerned with the fluid balance of the body.

The marked atonicity, ptosis, and, at times, the almost complete absence of peristaltic movements observed in the lower jejunum and ileum would tend to support the work of Robert-

son and Doyle ('35) on intestinal stasis in rats, which suggested to them that constipation may be a result of too low a level of potassium intake.

Whether or not the intussusceptions noted in our experiments were a part of the pathological findings due specifically to potassium deficiency is a question, but they would serve to indicate that peristalsis was impaired. The fewer intussusceptions found in the potassium deficient rats which received vitamin B may indicate that this condition was not a specific characteristic of the deficiency. There is the possibility, however, that the vitamin B solid carried sufficient potassium to partially prevent their occurrence. We have found no intussusceptions in hundreds of rats showing the extreme incoordination of acute vitamin B deficiency produced by diets containing adequate potassium. Moreover, the potassium deficient diet used in the present investigation contained ample vitamin B for the rat despite the extraction with acidulated water.

The early symptoms produced in young rats fed the magnesium deficient diet are essentially in agreement with those reported by McCollum and Orent ('31) and by Kruse, Orent and McCollum ('32 a) for the same animal. Certain differences should be mentioned, however. Their animals exhibited the following effect on the skin: "Within 3 to 5 days, average 4 days, all the exposed skin areas became vividly red from vasodilatation and hyperemia in the vascular bed." Our animals consistently developed a generalized hyperemia or vasodilatation of the skin. This condition was usually followed by the formation of circumscribed areas of erythema, hemorrhages, eschars, and exfoliation, especially prevalent on the dorsum of the back and on the flanks. In addition, there was occasionally seen in the later stages a pronounced edema in the pedal portions of the posterior extremities and in the nasal region. Greenberg and Tufts ('35 a, b), Cramer ('32), and Brookfield ('34) apparently obtained none of these symptoms in rats.

In general, our findings agree with those of Kruse et al. ('32 a) and Brookfield ('34) in regard to the tonic-clonic convulsive seizures and death. Our rats, however, usually exhibited only one seizure during an attack and did not always develop a convulsion when stimulated. Although some animals needed only a slight stimulus to induce a seizure, others required a prolonged stimulus; still other rats apparently passed into a seizure spontaneously. However, the greater number of animals died during the night. The postures assumed by them suggested that death may have resulted from a convulsion. Cramer ('32) observed no convulsions in rats fed a low magnesium diet, and Greenberg and Tufts ('35 a, b) obtained a convulsive seizure only after drastic stimuli were provided. The latter workers claim that increasing the vitamin G content of the diet entirely prevented the convulsive seizures.

Undoubtedly our basal diet had lost a good deal of its vitamin G content as a result of the prolonged extractive procedure used in its preparation. The group of rats on diet 232 plus vitamin B (table 2) was kept on experiment a total of 13 weeks. No abnormality ever appeared except the continued subnormal rate of growth; the rats averaged 121 gm. in weight at this time. This retarded growth may have been due to a suboptimum intake of vitamin G or it may have been due to a deficiency of other water-soluble factors.[2] The difficulty in obtaining magnesium or potassium free sources of the water-soluble vitamins should be considered in all work dealing with a deficiency of these elements. However, the fact that either the simple potassium and magnesium salts or the complex salt mixture prevented all the symptomatology and pathology was strong proof that these conditions in our

[2] The lacking factors were mainly supplied by brewer's yeast. Diet 232 supplemented with 0.5 gm. of brewer's yeast per rat daily gave a near normal gain; two female and two male rats had an average weight of 222 gm. after 8 weeks on experiment.

experiments were due to a deficiency of potassium or magnesium,[3] as the case might be.

Considering the spectacular nature of the symptoms obtained during magnesium deficiency in our rats, surprisingly little histopathology was encountered. Aside from the usual occurrence of changes in the skin, one-third of the animals studied microscopically (nine out of twenty-seven) evidenced degenerative changes in the livers. Just how much significance this liver degeneration has in magnesium deficiency is unknown. However, Brookfield ('34) also reported liver degeneration, but we are unable to determine from his data whether the same type of change was produced. Our findings are in disagreement with those of Brookfield ('34) and Cramer ('32) in showing no degeneration in the kidneys.

SUMMARY

1. Rats receiving a potassium deficient diet showed slow loss in weight, short fur-like hair, cyanosis, abdominal distention, and lethargy leading to coma and death (23 days average). Pathological changes occurred in the intestine, pancreas, kidneys and heart. Marked ascites was usually present. Hydrothorax and hydropericardium were an occasional occurrence.

2. Rats receiving a magnesium deficient diet showed a very subnormal growth in the early part of the experiment; part of this gain in weight was lost before the animals died. The animals consistently developed generalized hyperemia of the skin, which was followed by circumscribed areas of erythema, hemorrhages, eschar formation, and exfoliation. Edema of the extremities and nasal region was an occasional finding. An early hyperirritability, apparently progressive, led to

[3] Additional proof of the cause of the symptoms produced in rats fed the magnesium deficient diet (231E + K) was obtained. This diet was low in inorganic sulfur as well as in magnesium, because the former was supplied entirely as sulfate in $MgSO_4$. Sulfur in the same form, Na_2SO_4 in this case, was added to diet 231E + K in the same amount as was furnished by 4% of salts no. 186 or by 0.5% of $MgSO_4 \cdot 3H_2O$. The sodium content of the diet was maintained at a constant level by reducing the amount of $NaHCO_3$. No beneficial effect was observed on six rats fed this sulfur-supplemented magnesium deficient diet.

tonic-clonic convulsions (30 days average), and often to death (35 days average). Death was characterized by its suddenness and unpredictability. Considering the spectacular nature of the symptoms, surprisingly little histopathology was encountered. Aside from the usual occurrence of changes in the skin, one-third of the animals (nine out of twenty-seven) evidenced degenerative changes in the liver.

3. The symptomatology and pathology resulting from a deficiency of both potassium and magnesium were similar to those of potassium deficiency, except that the rats evidenced the early hyperirritability of magnesium deficiency.

4. No abnormalities other than a subnormal rate of growth were encountered when both magnesium and potassium, in the form of the simple salts or as a part of a complex salt mixture, were added to the basal diet. In each case the rate of growth was identical.

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PLATES

PLATE 1

EXPLANATION OF FIGURES

A Rat no. 6403. Potassium deficient diet. Photomicrograph showing the type of lesion found in the tissue intervening between the 'beaded' portions. Note the distention at the tips of the villi and the rarefaction of the submucosa at this point. A few cells have accumulated at the tips of the villi, and the epithelium at this point is edematous. Note also the relative scarcity of lymphocytes and phagocytes in the lumen of the intestine as compared with B. Hematoxylin-Triosin. 15 μ. Magnification $\times$ 160.

B Rat no. 6403. Potassium deficient diet. Photomicrograph showing the type of change consistently found in the 'beaded' portions of the lower jejunum and ileum. Note the markedly edematous and vacuolated epithelium at the tips of the villi, and their marked distention with lymphocytes and phagocytes. Numbers of these cells are also to be seen within the lumen of the intestine. Hematoxylin-Triosin. 15μ. Magnification $\times$ 160.

C Rat no. 6541. Potassium deficient diet. Photomicrograph showing the marked edema and rarefaction of the whole submucosa with uniform distention of the villi. This type of change was found in the same general areas as B. Hematoxylin-Triosin. 15 μ. Magnification $\times$ 160.

D Rat no. 6541. Potassium deficient diet. Photomicrograph of a section of the wall of the left ventricle of the heart showing the type of lesion consistently found. Note the marked scarring of the myocardium. Hematoxylin-Triosin. 15 μ. Magnification $\times$ 160.

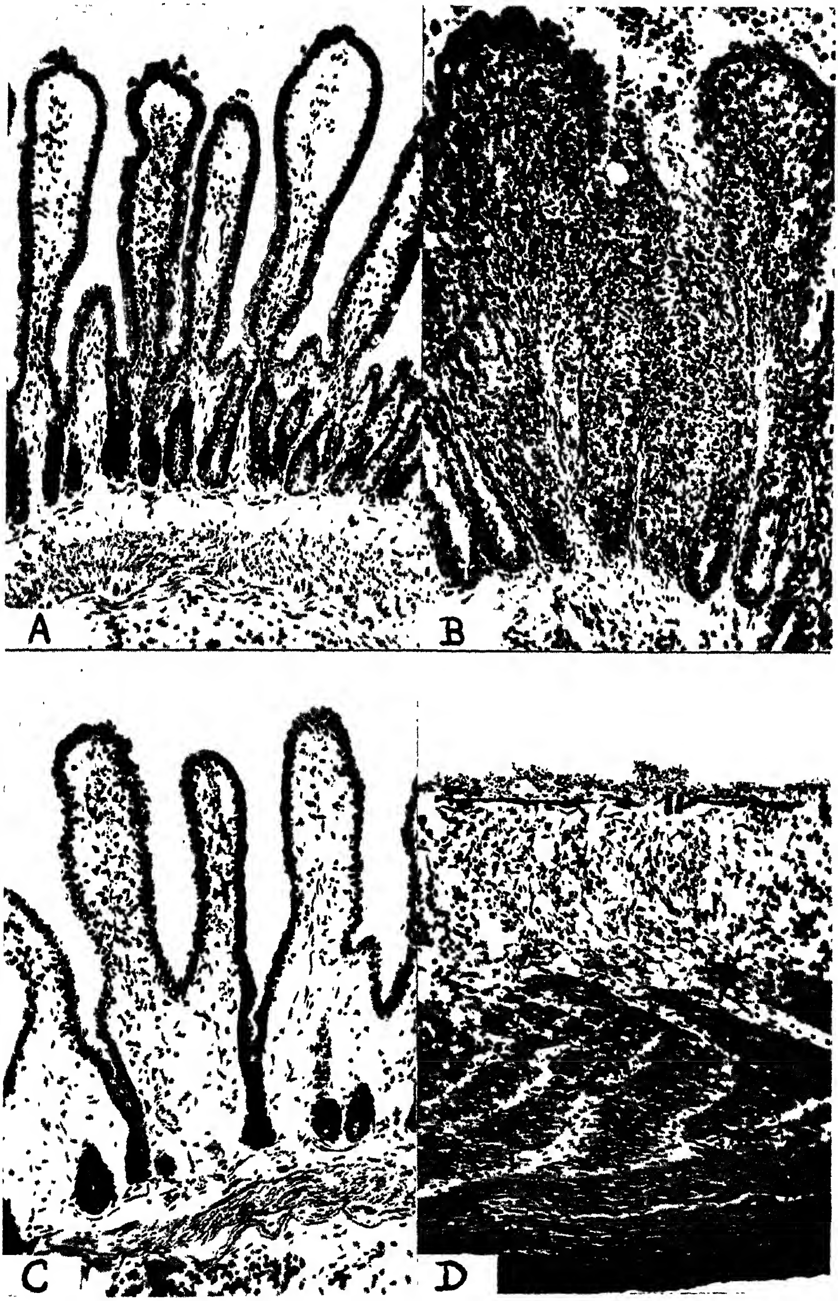

A
B
C
D

PLATE 2

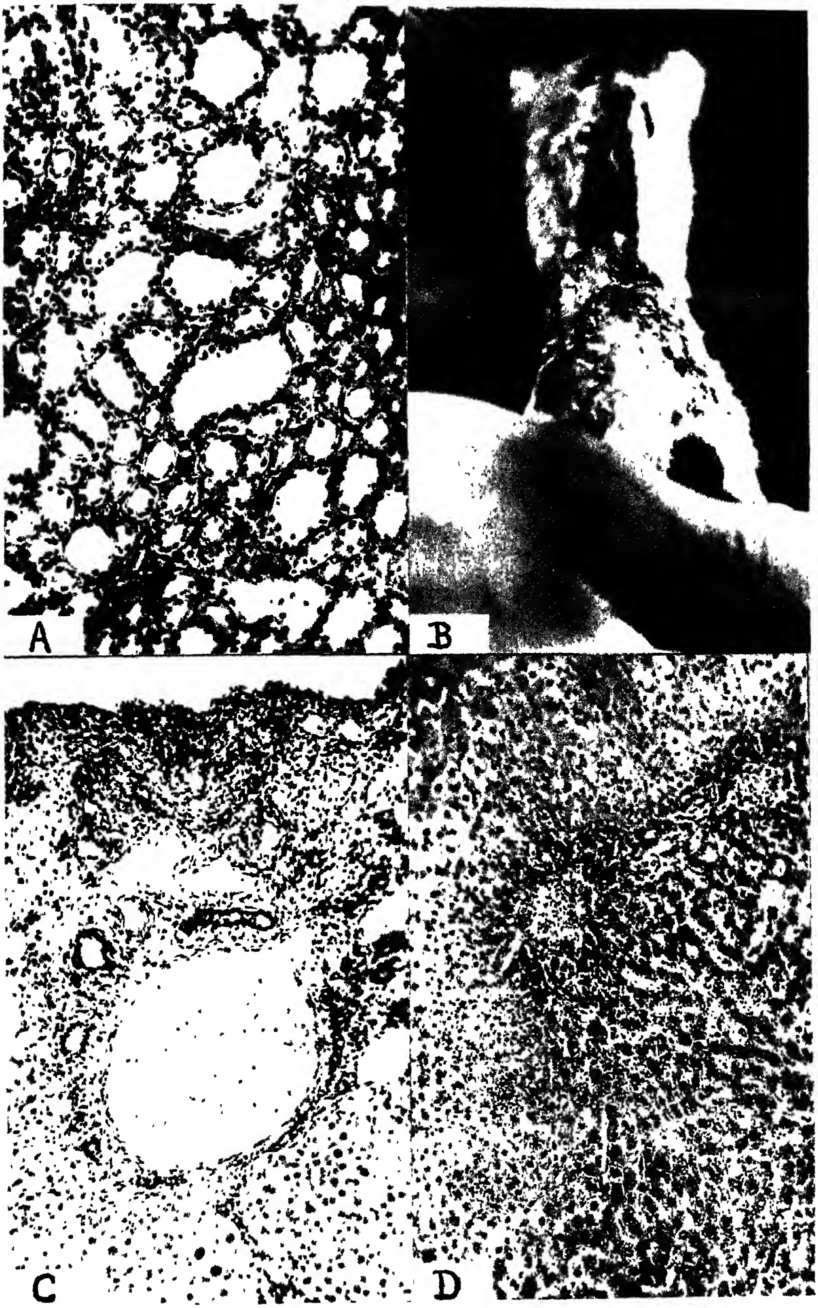
EXPLANATION OF FIGURES

A Rat no. 6551. Potassium deficient diet. Photomicrograph of a section of the kidney showing the marked dilatation of the tubules and necrosis of the tubular epithelium. Hematoxylin-Triosin. 15 μ. Magnification $\times$ 160.

B Rat no. 6731. Magnesium deficient diet. Photograph of a rat showing the marked escharotic lesions of the back and flanks.

C Rat no. 6558. Magnesium deficient diet. Photomicrograph of a section of liver showing the type of change found in those livers which had shown the cicatrizing type of lesion. Note the marked scarring and infiltration of lymphocytes and phagocytes. Hematoxylin-Triosin. 15 μ. Magnification $\times$ 160.

D Rat no. 6576. Magnesium deficient diet. Photomicrograph of a section of liver which had shown the mosaic type of lesion. Note the marked cellular destruction and the pycnosis of remaining nuclei. Hematoxylin-Triosin. 15 μ. Magnification $\times$ 160.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INDIVIDUAL VARIATIONS IN SUSCEPTIBILITY TO DIETARY DEFICIENCY [1]

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TWO FIGURES

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There are a good many clinical facts which suggest that people vary widely in their susceptibility to dietary deficiency disease. Not all of the crew of a scurvy-ridden ship come down, nor does everyone in a famine zone develop malnutritional edema. We have been impressed, furthermore, in general medical work by the fact that with certain gastro-intestinal disorders one patient will develop anemia, another pellagra, while a third may possibly have hypoproteinemia with edema. There seems to be some special susceptibility which determines just what clinical reaction, if any, will take place. It is well known that small animals vary greatly as regards tolerance to drugs and poisons but we have found no adequate quantitative data on variations in reaction to different diets. This problem was investigated in the following manner:

METHODS

Young mature male rats of the pure breed raised for many years in this laboratory were placed on a deficient diet consisting af casein 20% and a starch-lard mixture 80%. While not low in protein the ration was obviously defective in salts and vitamins even though the constituents were not chemically purified. It was known to us from previous experience that progressive malnutrition results with this diet, but loss of weight is slow enough to be roughly comparable to human deficiency disease. The animals were caged in groups and an

[1] Supported by a grant from the Rockefeller Fluid Research Fund.

excess of the diet was offered; no attempt was made to measure the individual intake. Weights were recorded every few (usually 2) days and clinical evidences of malnutritional disease were looked for.

RESULTS

Fourteen rats were used. During period 1 (table 1) they were given the defective diet for 39 days. Initial and final weights, grams lost and percentage of weight lost are shown. It is evident that there were great variations in the reaction of individuals to the diet even under practically identical conditions. No clinical evidences of malnutritional disturbances other than loss of weight were observed.

Having established the fact of sensible variations in weight loss under the conditions of our experiment, we desired to find out if these variations were haphazard and of no significance or whether the degree of weight loss was a characteristic of the individual rat. The animals were therefore returned to the stock diet and there was a very rapid gain in weight (figs. 1 and 2). After 22 days the defective diet of casein-starch-lard was resumed for a second period of 49 days, constituting period 2 (table 1). Loss of weight again occurred and again there were great individual variations. More interesting, however, is the fact that in general the rats which lost most during period 1 again lost most in period 2, and vice versa. The probability, though not yet proved, was strong that some definite peculiarity of individual rats determined whether weight loss would be slight or great and that the results were not haphazard.

Another repetition of the experiment was therefore carried out after the rats had been restored by eating the stock ration for 31 days, but this time, to put the matter to a more rigorous test, an entirely different type of defective diet, namely raw carrots, was used. The results of period 3 are shown in table 1. Here for the third time it is seen that on the whole those rats which lost most in the two experiments with the casein-starch-lard diet did so again on the carrot diet. In table 2 an attempt is made to show this graphically. In the

TABLE 1

Summary of data on weight loss of rats on deficient diet

PERIOD 1—39 DAYS					PERIOD 2—49 DAYS					PERIOD 3—38 DAYS				
Rat no.	Weight at start	Weight at end	Loss	Per cent of original weight	Rat no.	Weight at start	Weight at end	Loss	Per cent of original weight	Rat no.	Weight at start	Weight at end	Loss	Per cent of original weight
	gm.	gm.	gm.			gm.	gm.	gm.			gm.	gm.	gm.	
25	182	150	32	82.4	38	214	168	46	78.5	38	224	182	42	81.2
34	196	156	40	79.6	23	210	162	48	71.1	36	226	176	50	77.8
23	186	144	42	77.4	25	210	158	52	75.2	65	236	182	54	77.1
39	186	142	44	76.3	34	214	160	54	74.76	23	226	174	52	76.9
56	182	138	44	75.8	39	206	154	52	74.75	25	226	174	52	76.9
38	202	152	50	75.2	37	200	148	52	74	54	216	166	50	76.8
54	182	136	56	74.7	59	200	142	58	71	34	226	170	56	75.2
36	204	152	52	74.5	56	202	142	60	70.29	50	216	158	58	73.1
52	196	142	54	72.4	36	242	170	72	70.24	59	224	162	62	72.3
37	198	142	56	71.7	54	204	142	62	69.6	39	240	168	72	70.0
51	182	126	56	69.2	65	238	162	76	68.0	51	216	150	66	69.4
50	178	122	56	68.2	51	192	124	68	64.5	52	240	164	76	68.3
65	196	128	68	65.3	52	224	136	88	60.7	37	226	152	74	67.2
59	182	118	64	64.8	50	206	112	94	54.3	56	210	140	70	66.6

column headed 'period 1' the rats are arranged in order of weight loss, with a separation into first and second divisions. In column 2 the rats are again arranged in sequence of weight loss, preserving the same numerical notation. It is seen that

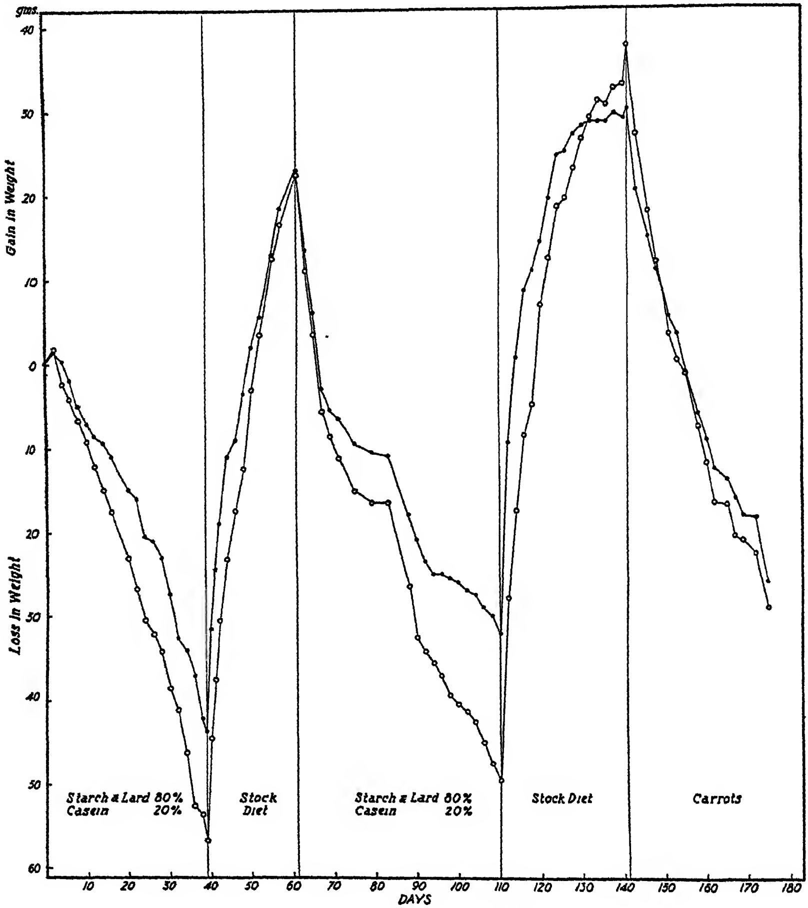

Fig. 1 Average variations in weight of rats of first division (dots) and of second division (circles).

with two exceptions the top division remains unchanged and so too in the third period, with the exception of the jump of rat no. 65 from eleventh to third place and of rat 36 from ninth to second place, there is little alteration of sequence.

A mathematical calculation shows a probability of one in sixty-four of these events being the result of chance.

In figure 1 are shown average weight curves of rats 25, 34, 23, 39, 56, 38 and 54 (dots) and of rats 36, 52, 37, 50 51, 65, 50 (circles). Both curves start from the same point but the changes of weight are recorded in grams and not as percentage variations. It is seen that the rats of the second division consistently lost more weight in each of the three experimental periods than those of the first division.

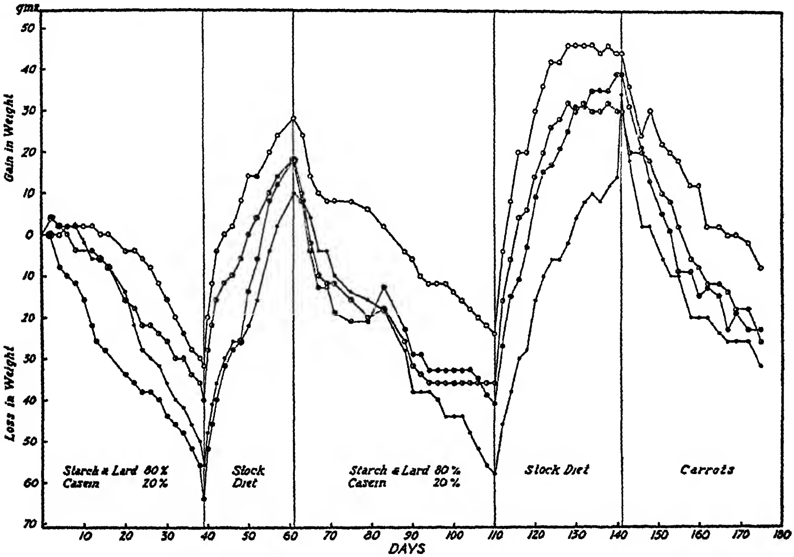

Fig. 2 Individual weight curves of four rats—no. 25 (circles), no. 34 (half circles), no. 51 (dots), no. 59 (crossed circles).

Figure 2 shows the detailed weight changes of four rats. It was technically impossible to chart the entire series on one map; however, the specimen curves give an adequate idea of the course of events in individual rats.

SUMMARY AND CONCLUSIONS

When a series of rats of the same breed and of approximately the same age are placed on a defective diet there are great individual variations in weight loss. Repetitions of the

experiments after weight loss has been restored by a normal diet, show that on the whole the rats which lost most weight in the first instance do so again and vice versa. Resistance to weight loss on a defective diet seems therefore to be in some

TABLE 2

Rats arranged in series according to degree of weight loss in the three periods of the experiment

1	2	3
25	38	38
34	23	36
23	25	65
39	34	23
56	39	25
38	37	54
54	59	34
36	56	50
52	36	59
37	54	39
51	65	51
50	51	52
65	52	37
59	50	56

cases a characteristic of the individual and not a matter of chance. These results are shown to be statistically valid. Further studies on the explanation of this phenomenon are in progress.

'LATENT DEFICIENCY' IN RATS: VARIATIONS IN WEIGHT LOSS ON REPEATED FEEDING OF A DEFECTIVE DIET

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EIGHT FIGURES

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In the course of previous experiments we studied the curve of weight loss of rats on a defective diet consisting of 20% casein and 80% starch-lard mixture. When rats which had lost from 20 to 40% of their body weight were restored to 'normal' by a sojourn on an adequate stock ration [1] and were then placed again on the defective diet it was found that in the second instance weight loss occurred much more rapidly than in the first. This held true in every case, even though the original speed of weight loss varied considerably with different rats. Two sample curves are given in figure 1. The solid lines show weight loss during the first period on the defective diet, the broken lines represent the second period. In both cases the initial weight is taken as 100 and the changes are expressed in actual grams lost per day. The intermediate period of 22 days on stock diet, during which weight was fully restored, is omitted. It is seen that whereas rat 59 lost much more rapidly in the first instance than rat 25, each lost more rapidly in the second period of defective diet than in the first. Another fact shown by these curves is that, whereas weight loss was more rapid the second time, this rapid loss was most striking during the first 10 days or so of the second period

[1] Corn starch, casein, lard, cod liver oil, salt mixture and alfalfa. See Addis, McKay and McKay. 1926. J. Biol. Chem., vol. 71, p. 139.

of defective diet. Indeed the decline slows subsequently so that after a longer period (30 to 40 days) there may not be much difference in the total weight loss during the first and second periods of defective diet. This is again shown in figure 2, a composite curve of fourteen rats.

Before going further it was necessary to be sure that the rapid weight loss during the second sojourn on defective diet

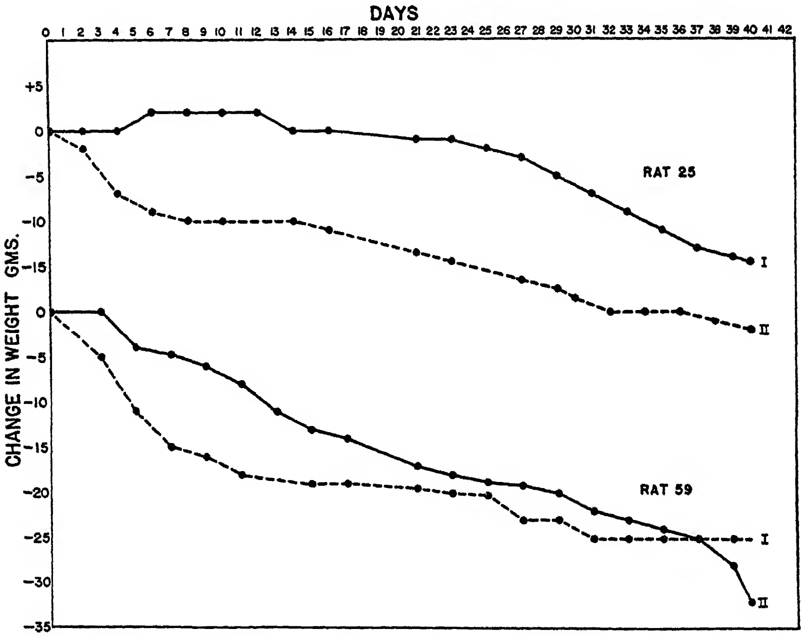

Fig. 1 Curves of weight loss of two rats on defective diet. First trial (I), second trial (II).

was not due simply to the fact that the rats were older or to differences in external conditions such as season or environmental temperature. The following experiment was therefore carried out:

Sixty young but mature female rats were divided into two groups of thirty each as nearly alike as possible. The average weights of the two groups at the start of the experiment were, respectively, 168.3 gm. and 170.5 gm. Group I remained on

the stock diet as controls, the second group was placed on the defective diet for 39 days. At the end of this period the average weight had declined to 129.9 gm. whereas that of the controls was 191 gm. Group II were then replaced on the stock diet and rapidly gained weight so that after a period of 20 days they averaged 194.1 gm. At this time the average weight of the control group I which had so far been continuously on stock diet was 201 gm. Both groups were now

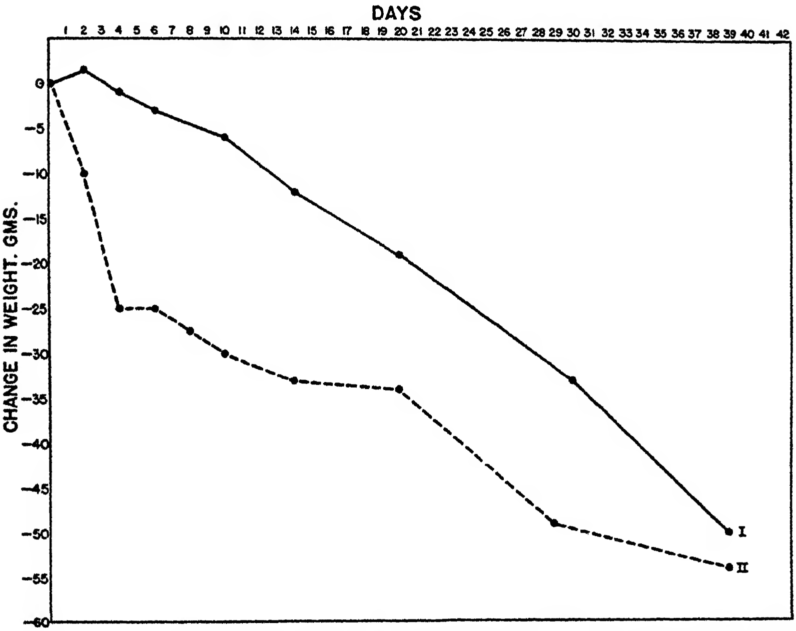

Fig. 2 Composite weight curve of fourteen rats during first and second trials on defective diet.

placed on the defective diet and as one sees from figure 3 group II lost weight not only more rapidly than during the first sojourn on defective diet but more rapidly than the control group. Furthermore, the curve of weight loss of group I is almost identical with that of group II during the first trial on defective diet. At the conclusion of the experiment autopsies were done on fourteen rats from each group. Special search was made for the usual evidences of vitamin deficiency

such as lesions or dystrophies of eyes, skin, hair, claws, nose, tongue, tail and internal organs, especially the gastro-intestinal tract. The rats were all active and healthy looking at the time of autopsy, the fat stores were still abundant and no macroscopic lesions were discovered. Rapid weight loss in group II was therefore not due to gross disease or to age or environmental influences.

It seems established then that the original experience on the defective diet modifies the rat in such a way that weight is lost more rapidly when the experience is repeated. The

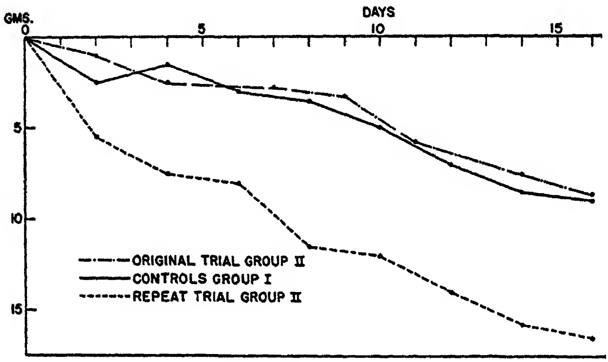

Fig. 3 Control experiment to rule out accidental influences in weight loss. (For explanation see text.)

attempt was therefore made to work out some of the quantitative aspects of this phenomenon.

Experiment 1

When rats have lost 20 to 40% of body weight as a result of the defective diet and then are placed on a normal diet, after how long a sojourn on such normal diet does rapid weight loss still occur if defective diet is again fed? Does the effect of the first experience persist through the entire life of the rat or does it wear off as time goes by?

Procedure. Six groups of five each of young mature female rats were selected so that the average weight of each group was practically the same (table 1). The rats were placed for

50 days on the defective diet of casein 20% and starch-lard 80%. They were then returned to stock diet for varying lengths of time, after which they were re-tested on the defective diet. Group I was on stock diet for 11 days before the second test, group II for 21 days, group III for 31 days, group IV for 41 days, group V for 62 days, group VI for 82 days. The results are exposed in table 1 and figure 4. Considering from our present standpoint only the critical period

TABLE 1

Experiment 1. Summary of data on weight loss of various groups of rats during first and second trials on defective diet

GROUP	ORIGINAL DEFECTIVE DIET					REPEATED DEFECTIVE DIET				
	Weight	Loss 10 days		Loss 20 days		Weight	Loss 10 days		Loss 20 days	
		Grams	Per cent	Grams	Per cent		Grams	Per cent	Grams	Per cent
1	180	0.4	0.2	9.5	5.3	194.8	8.4	4.3	11.1	5.7
2	179.6	2.4	1.3	11.0	6.1	204.8	15.9	7.8	20.0	9.8
3	178.8	0.2	0.1	6.0	3.4	206.8	12.4	6.0	14.0	6.8
4	182	2.8	1.5	10.2	5.6	214.4	13.4	6.2	22.0	10.3
5	180	Gain 1.6	Gain + 0.9	6.4	3.6	209.6	11.6	5.5	18.8	9.0
6	183	Gain 3.0	Gain + 1.6	3.9	2.1	228.0	15.4	6.8	23.2	10.2

of the first 10 days on defective diet, it appears that in every instance weight was lost much more rapidly during the second test period than during the first. Even when the interval on stock diet after the first feeding of defective diet was as long as 82 days rapid loss of weight still occurred on the repeated defective diet. It is evident therefore that even though these rats appeared perfectly normal when placed for the second time on defective diets, a latent defect still remained as a dormant legacy of the original insult.

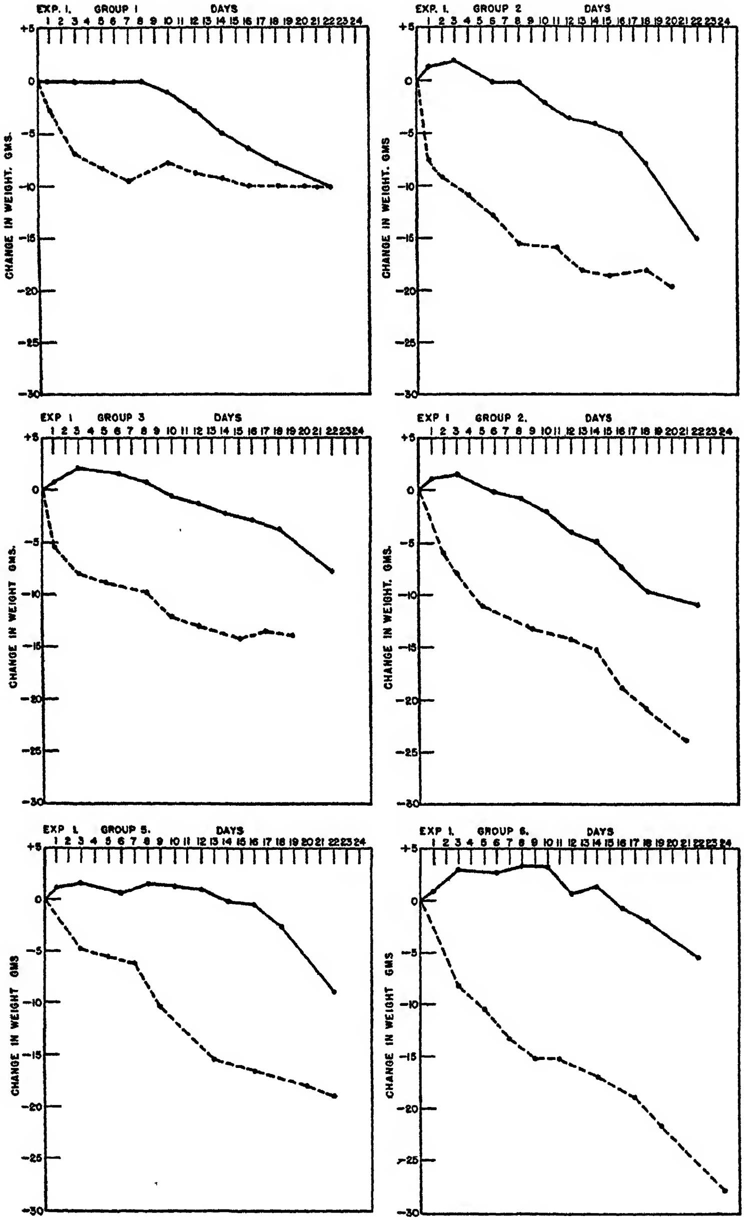

Fig. 4 Graph of data from experiment I (see text). The solid lines are weight curves during first trial on defective diet, the broken lines represent the second trial.

Experiment 2

How long an original sojourn on defective diet is necessary to secure the rapid weight loss on repeated defective diet?

Procedure. Five groups of rats (groups II to VI) similar to those used in experiment 1 were placed on the defective diet. A sixth group (I) were used as weight controls. Group II were placed on defective diet for 10 days only, then on stock diet until their weight had returned to that of the controls (1 day), and then for the second time on defective diet.

TABLE 2

Experiment 2. Summary of data on weight loss of various groups of rats during first and second trials on defective diet

DURATION OF FIRST STAY ON DEFECTIVE DIET	GROUP	ORIGINAL DEFECTIVE DIET					REPEATED DEFECTIVE DIET				
		Weight	Loss 10 days		Loss 20 days		Weight	Loss 10 days		Loss 20 days	
			Grams	Per cent	Grams	Per cent		Grams	Per cent	Grams	Per cent
days											
10	2	188.4	2.4	1.3			192.8	5.6	2.9	17.0	8.8
20	3	188.8	1.2	0.6	5.2	2.8	204.0	11.0	5.4	20.4	10.0
30	4	187.2	3.6	1.9	6.8	3.6	203.2	7.6	3.7	14.8	7.3
40	5	185.2	+1.2	+0.6	3.8	2.1	205.2	26.8	13.1	38.6	18.8
51	6	186.0	1.6	0.8	4.6	2.5	208.8	11.8	5.6	19.2	9.2
	1	208.5	7.7	3.7	17.2	8.2					

Similarly group III were on defective diet 20 days, stock diet until control weight was regained (4 days) and then for the second time on defective diet. Group IV was on defective diet 30 days, group V 40 days and group VI 60 days. The results are shown in table 2 and figure 5. It is seen that an original sojourn of only 10 or 20 days was sufficient to lead to weight loss more rapid than that of any control group when the rats are tested for the second time. Longer periods on defective diet (groups IV and V) did not serve materially to increase weight loss on repeated exposure.

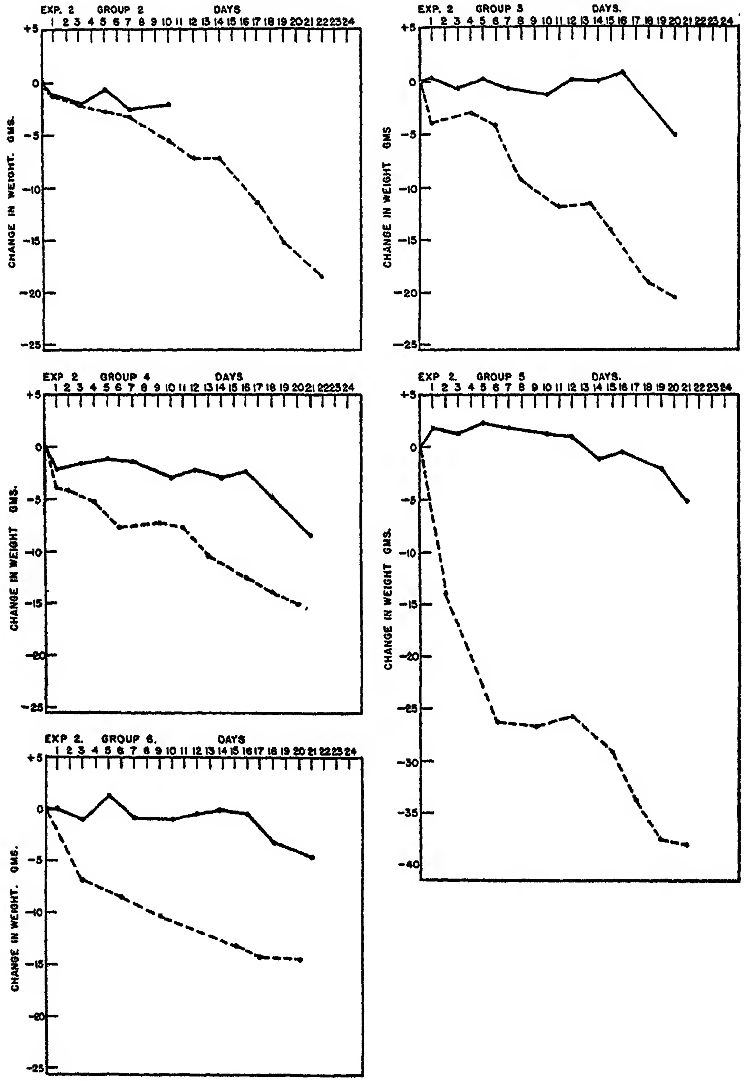

Fig. 5 Graph of data from experiment 2 (see text).

DISCUSSION

These experiments show in brief, that if normal adult rats are subjected to a defective diet and are then restored to apparently normal health and weight by stock diet, even after long periods a second sojourn on defective diet is followed by a prompt and rapid loss of weight invariably greater than that obtained during the original feeding of the same diet. There has been established what for the sake of convenience we shall term a state of 'latent deficiency.'

The following possibilities suggest themselves as explanations of this phenomenon: First of all there might be tangible anatomical lesions, perhaps the result of vitamin deficiency, perhaps from other causes, which are responsible for poor appetite or inadequate absorption of food. No such lesions were found, as we have pointed out above. Next one had to consider the possibility that the rats simply did not like the defective diet and that when they were placed on it for the second time they recognized it as having been unpalatable previously and therefore ate less. This does not, however, seem an altogether satisfactory explanation. Secondary rapid weight loss in no way ran parallel with the interval between the two feedings of defective diet; even after 80 days on stock diet (fig. 4) such secondary rapid loss was very striking. It seems improbable that mere distaste for the diet would persist over such a long portion of the rats' life, especially since the ingredients are all those which enter into the stock diet which rats eat indefinitely with apparent relish. Finally comes the question of whether loss of weight is due to lowered food intake which in turn is the result of some state of deficiency produced by the original sojourn on defective diet. We have found it technically impossible to measure accurately the rats' daily intake of the present diets; it may be accepted, however, as reasonable that loss of weight in animals without gross disease is usually associated with diminished intake. The problem is whether such diminished intake is a specific effect of a deficiency state. There is in the literature considerable support for this point of view. Animals on diets

lacking in vitamin B, for example, can be made to eat more and to gain weight by furnishing the vitamin separately from the diet so that palatability is not altered (Karr, '20). Furthermore, rate of gain has been shown to run parallel to some extent with the amount of the accessory substance which is furnished (Osborne and Mendel, '17). Certain investigators present experiments (Sure, Kile and Smith, '32) which they believe prove that vitamin B possesses the physiological function of stimulating growth per se, unrelated to food intake.

In an effort finally to settle the point the following experiments were carried out. Four rats with an average weight of 162 gm. were given each day 2 cc. of olive oil and 2 cc. of 50% glucose by stomach tube. A Luer syringe attached to a small cannula was found satisfactory, the procedure being carried out under light ether anesthesia. The tube feedings were given for 14 days at the end of which time the average weight was 141 gm. The rats were then placed on stock diet for 10 days and the average weight returned to 166.5 gm. Tube feeding was then repeated. Figure 6 shows the composite weight curves during the first and second periods of tube feeding, and figure 7 shows the events in an individual rat, no. 32591. No lesions were found at autopsy in this animal. In a second experiment five rats were tube fed as above for 5 days and then placed on the defective diet of casein and starch-lard mixture for 34 days. They were then restored to 'normal' by 2 weeks of stock diet when the original experiment was repeated. Four control rats of about the same weight as the test animals were tube fed and treated in the same way during this second experimental period. The average weight of test rats at the start of the second trial of tube feeding was 202 gm., and of the controls 200 gm. The events are shown in figure 8. It is seen in both experiments that even with the food intake controlled by tube feeding, a second period of defective diet results in more rapid weight loss than the first trial, although the discrepancy is not as great as when food is simply placed in the cage.

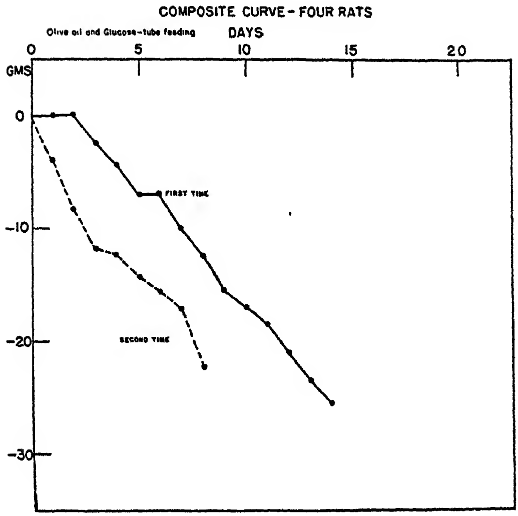

Fig. 6 Curve of weight loss in tube feeding experiment (see text.)

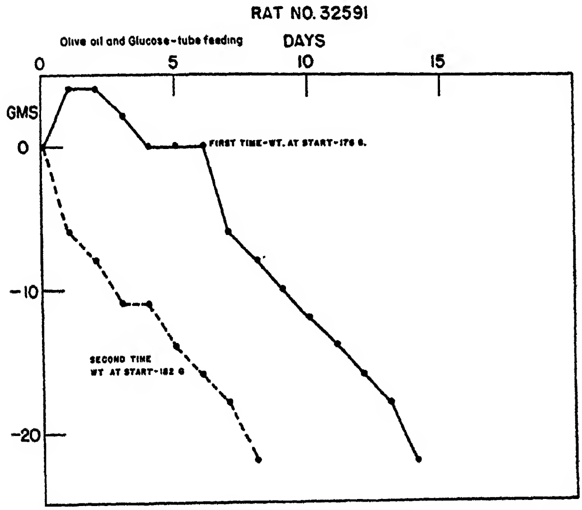

Fig. 7 Curve of weight loss in tube feeding experiment (see text).

It seems clear therefore that the rapid secondary weight loss described in our experiments is not an artifact but is associated with some real alteration in the animal which has been produced by the original sojourn on defective diet. There has been established what may perhaps be called a state of 'latent deficiency.'

It is known that a previous state of clinical deficiency from which the animal appears to have recovered may occasionally

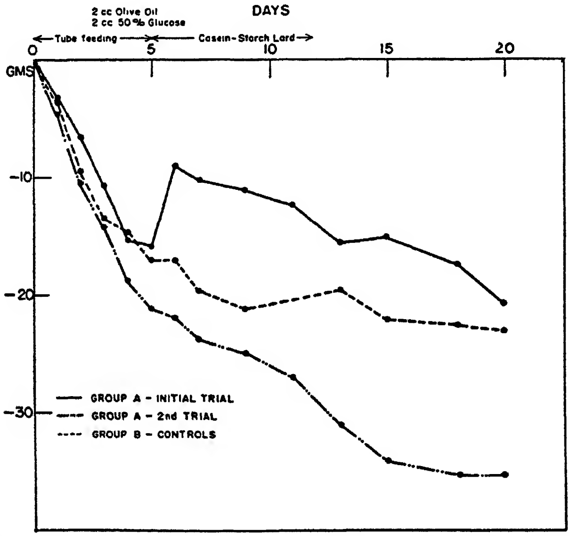

Fig. 8 Curve of weight loss in tube feeding experiment (see text).

be followed by certain lasting effects such as sterility. Baumann, Riising and Steenbock ('34) made quantitative studies of absorption and storage of vitamin A in the rat. Of interest in connection with our work, they point out how readily vitamin A stores may be depleted and the difficulty in restoring large reserves. Rats in which no A could be demonstrated might appear perfectly well and continue to grow for several weeks before clinical xerophthalmia appeared. King and Menten ('35) report that guinea pigs partially depleted of vitamin C reserves but without showing external signs of scurvy succumbed after injection of diphtheria toxin in half

the time that controls survived. Similar studies have been made in man such as that of Jeans and Zentmire ('36) who concluded, using dark adaptation as a test for vitamin A inadequacy, that 26 to 79% of children in Iowa had a latent deficiency.

SUMMARY

Rats which have lost weight as the result of a defective diet and have then been restored to 'normal' by stock ration show a more rapid weight loss if now placed for a second time on the same defective diet. This 'secondary rapid weight loss' may occur after as long an interval as 80 days between the first and second periods on defective diet. The nature and significance of the phenomenon are discussed, and it is pointed out that it is not due to anatomical lesions or to accidental conditions but must be ascribed to some real alteration in the status of the animals.

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FURTHER STUDIES ON THE GROWTH PROMOTING FACTOR ASSOCIATED WITH SUMMER MILK [1]

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ONE FIGURE

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In 1934 it was shown by Elvehjem, Hart, Jackson and Weckel ('34), Stirn, Elvehjem and Hart ('35) that milk produced by cows on a regular winter ration was markedly inferior in nutritive value to milk produced by cows that had access to summer pastures. Properly prepared rats on mineralized summer milk grew more than 4 gm. per day over a 6-week period, while rats on mineralized winter milk grew only about $2\frac{1}{2}$ gm. per day.[2] In a recent note the authors ('36) pointed out that the feeding of 3 cc. of grass juice per day to rats on winter milk stimulated growth to more than 4 gm. per day, a rate comparable to that obtained on summer milk.

Thus the seasonal changes in the nutritive value of milk can be related directly to the presence or absence of a factor, or factors, in the forage ingested by the cows. The green succulent grass consumed during certain periods of the summer carries an amount of the factor in question which is sufficient, not only for the body needs, but also for transmission into the milk. During the winter months, the cow

[1] Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

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[2] 'Properly prepared' rats refers to special care taken to prevent the young rats from eating the mother's ration and mineralized milk is milk to which specific quantities of iron, copper and manganese are added to supply mineral deficiencies in the milk.

must rely mainly upon dried fodder which has lost most of its potency in respect to the factor under discussion through preparation and storage. The growth promoting property of the milk may be maintained until the stores of the factor in the animal's body are exhausted, but then the amount in the milk decreases.

We should point out that our work has been with cows maintained in a region where common practice dictates that they receive dry fodder supplemented with corn silage at least 6 months of the year. In other areas, where the winter period is shorter, the nutritive change in the milk which we have noted may not be so drastic. However it is possible to decrease definitely the growth promoting value of milk at any time of the year by placing cows on a dry ration for several months.

From a practical point of view, several interesting questions arise. First we must consider the possibility of producing a milk of summer quality the year around. The possible use of A.I.V. silage[3] and forage preserved by other methods for the purpose of improving the nutritive quality of milk is now in progress in this laboratory and preliminary results have been published (Peterson et al., '35). Another question is the preservation of the growth promoting quality of milk from the time of production until consumption. Work on the effect of pasteurization has been published (Elvehjem et al., '34) and studies on canning and other methods of processing will be published in another paper. The other possibility is the use of foods rich in this factor as supplements to milk of known decreased nutritive value. Studies on this question as well as the relation of the factor to known vitamins will be presented in this paper.

In 1935 in a paper dealing with the nutritional anemia produced in rats restricted to a diet of goats' milk (Kohler, Elvehjem and Hart, '35), it was reported that, although the anemia was corrected by the addition of iron and copper,

[3] A.I.V. silage is the name given to green forage preserved with mineral acids according to the method originated by A. I. Virtanen.

growth on the mineralized goats' milk was very poor. In many cases the rats, after a short period of growth, lost weight and finally died. It was further demonstrated that this condition was completely corrected by the addition of 0.25 gm. of dried hog brain per day to the mineralized goats' milk fed ad libitum. Growth rates as high as 5 gm. per day were obtained with rats on goats' milk by the use of this supplement. Other supplements such as pure vitamin $B(B_1)$, cod liver oil, and yeast showed little or no activity. Dried whole liver produced a definite response but was not as potent as the brain.

At first it was tentatively assumed that the limiting factor of goats' milk and that of cows' milk were the same, but it soon became evident that this was not the case, since brain, which produced such striking results as a supplement to goats' milk, has proved to be a poor supplement for winter cows' milk.

The purpose of the work presented in this paper was to demonstrate the identity or non-identity of the factor found in grass juice with known vitamins.

EXPERIMENTAL

Certain precautions were taken previous to the experimental period to reduce the possibility of storage by the young rats of the factor involved. When the rat pups were 10 days old, the litters together with the mothers were placed in individual cages with wire screen bottoms. The only food in the cages was the basal winter milk. The mothers were removed once a day to receive the grain ration used for the stock colony. Thus the ingestion of the growth factor was limited to that in the mother's milk. In spite of these precautions we have found certain litters of rats to give fair growth even on some of the milks known to have a reduced nutritive value.

The rats were weaned at 3 weeks of age and started on the experiment immediately, each rat being placed in an individual cage. The supplement to be tested was placed

in the feed dish in the morning together with a little milk. Later in the day, when the rat had completely consumed the supplement and milk, the dish was filled with milk so that ad libitum feeding was assured. The rats received their iron, copper and manganese in the forms of ferric pyrophosphate, cupric sulfate and manganous sulfate, respectively. These minerals were administered as follows:

First week: 0.5 mg. Fe + 0.05 mg. Cu per rat daily
Second week: 0.5 mg. Fe + 0.05 mg. Cu + 0.04 mg. Mn per rat daily
Third to sixth week: 1.5 mg. Fe + 0.15 mg. Cu + 0.15 mg. Mn per 100 cc. of milk

For the first 2 weeks, the minerals were given as solutions with the supplements in the morning. During the rest of the experiment, the minerals were added to the bulk of the milk as indicated. The duration of the experiments was 6 weeks and the animals were weighed weekly. The milk was brought daily from the dairy barn in aluminum pails, the portion reserved for afternoon feeding being stored in a refrigerator.

Milks from individual cows on winter barn rations were used as basal diets for the supplement work. Some of these cows had received no other ration since they were weaned, a duration of about 3 years. A typical ration, that of cow no. 6 was as follows:

18 pounds grain	37 corn meal 37 ground oats 26 gluten meal 1 iodized salt
25 pounds corn silage	
7 pounds timothy hay	

No consistent difference in growth promoting quality was found between the milk from cows on this ration and that from cows on a comparable ration containing alfalfa instead of timothy hay (table 1). In fact the limited data which we have indicate that the rats on the milk from the cows receiving timothy hay grew slightly better than those receiving the milk from the cows on alfalfa hay. Recent results (unpublished data) indicate that the potency of a hay in the growth factor, with which we are here concerned, may vary

considerably depending upon the conditions under which it was grown, the stage of growth when cut, and the method of drying. Thus, it is quite possible that if all three of these variables were optimally controlled, a high quality of milk might be produced even on a dry ration. However, hay produced under ordinary farm conditions contains little or none of the active principle, hence the seasonal variation in the nutritive quality of commercial milk (Elvehjem, Hart et al., '34).

TABLE 1

The growth averages of rats on mineralized milk from cows of different breeds and receiving various dry rations

COW	BREED	ROUGHAGE	MALE RATS		FEMALE RATS	
			No.	Daily gain in weight	No.	Daily gain in weight
				gm.		gm.
No. 2	Holstein	Timothy	2	2.59	3	2.35
No. 5	Holstein	Timothy	20	2.78	17	2.15
No. 6	Holstein	Timothy	10	2.61	12	2.04
No. 10	Holstein	Alfalfa	3	2.33	1	1.93
No. 11	Holstein	Alfalfa	9	2.02	7	1.76
No. 18	Holstein	Alfalfa	1	2.26	3	1.74
No. 909	Ayrshire	June grass and alfalfa	7	2.76	5	2.07
No. 911	Ayrshire	June grass and alfalfa	7	2.59	5	2.03
Christine	Guernsey	Alfalfa	10	2.55	7	2.40

The growth promoting value of milk varies considerably with individual cows. These individual variations generally overshadow effects of breed or ordinary changes in winter rations. Growth averages of rats on mineralized milk from cows of various breeds on dry rations are given in table 1. In spite of the different variables the growth falls quite consistently between 2.0 to 2.8 gm. per day.

The list of supplements tried and growth responses obtained when they were fed in addition to mineralized winter milk are given in table 2. In each case, the increase in growth of the rats on the supplemented diet over that obtained for

rats from the same litter on the basal mineralized milk alone are given in terms of daily gain. Although male and female rats were used for the supplement studies, the table includes records for male rats only since, as is shown in table 1, females do not show the marked difference that the males do. The figures given represent the average of three or four rats in each case. The average daily gain is also included for those supplements showing activity so that the growth may be compared with previous data. It is readily seen that those sup-

TABLE 2

The effect of various supplements as additions to mineralized winter milk (male rats only)

SUPPLEMENT	LEVEL FED (PER DAY)	GAIN OVER CONTROLS (GM.) PER DAY)	GAIN IN WEIGHT (GM.) PER DAY)
Cod liver oil	0.1 gm.	0.08	
Orange juice	1.0 cc.	—0.52	
Defatted wheat germ	1.0 gm.	0.27	
Brewer's yeast	0.1 gm.	0.14	
Brewer's yeast	0.5 gm.	0.49	
Dried hogs' brain	0.25 gm.	0.55	
B_2 concentrate	≅ 0.5 gm. liver extract	0.55	
Fresh lawn grass	3.0 gm.	1.68	3.91
Grass juice	0.5 cc.	0.91	
Grass juice	3.0 cc.	1.69	4.48
Dried oat grass no. 180	0.6 gm.	1.36	3.98
Dried oat grass no. 223	0.6 gm.	0.55	3.17
Liver extract sample 1	0.2 gm.	1.38	
Liver extract sample 1	0.5 gm.	1.23	3.40
Liver extract sample 2	0.5 gm.	0.67	
Rice bran	0.2 gm.	1.29	3.77

plements supplying ample amounts of the better-known vitamins produced no significant increase in growth. The results with cod liver oil definitely eliminate vitamins A and D. Results given in a previous paper (Elvehjem, Hart et al., '34) demonstrated that the rats were ingesting sufficient amounts of vitamin A from the winter milk itself.

Since rats synthesize vitamin C, it was unnecessary to test this factor, but nevertheless orange juice was fed to eliminate any other factors it might carry. Thus vitamin P, the

factor recently reported by Rusznyák and Szent-Györgyi ('36) cannot be concerned in this problem.

Yeast fed at a fairly low level (0.1 gm. per day) produced no appreciable response. Vitamin B(B_1) assay of this yeast (unpublished data) showed that only 0.025 gm. was required per day to give optimum growth on a vitamin B low ration. Hence the rats on winter milk could not have been suffering from an insufficient intake of vitamin B. Additional evidence against a possible B deficiency is the poor growth obtained with the defatted wheat germ (Vio Bin)[4] when fed at a level of 1.0 gm. per day. Assay of this product showed that this level supplied about ten times the amount of vitamin B needed for optimum growth.

Since Birch, György and Harris ('35) found that 0.2 gm. of yeast per day supplied ample flavin and vitamin B_6 for a rat, several rats were given a higher level of yeast. Five-tenths of a gram per day was fed to insure against variations in yeast. Although this level of yeast did produce a slight effect, growth did not approach normal values (e.g., that obtained on summer milk). These results indicate at least that the limiting factor of winter milk is not flavin or vitamin B_6. Also none of the symptoms of either deficiency was observed in any of the rats on the basal winter milk.

Another factor which was ruled out by the poor growth response produced by 0.5 gm. yeast per day is the 'alcohol-ether precipitate factor' from liver extract. Elvehjem, Koehn and Oleson ('36) have shown that 4% of yeast supplies enough of this factor to produce a maximum growth response, and 0.5 gm. per day is equivalent to about 10% of the rat's intake of food calculated on the dry basis. Further proof of the non-identity of this factor with the limiting factor of winter milk is furnished by the fact that 20 cc. of winter milk per day is all that is needed to produce optimum growth in rats on the 'alcohol-ether precipitate factor' deficient basal ration used, while rats on mineralized winter milk alone consume 35 to

[4] Vio Bin is a wheat germ product which has been defatted and was kindly supplied by the Vio Bin Corporation, Chicago.

50 cc. per day. Thus they were getting in the basal milk diet about twice as much of the 'alcohol-ether precipitate factor' as is necessary for optimum growth.

Defatted wheat germ has been shown to be a good source of vitamin B_4 in chicken work in this laboratory (Kline et al., '36). Since it is very difficult to produce a B_4 deficiency in rats, it is probable that the B_4 requirement of the rat is very low, and therefore 1 gm. of defatted wheat germ per day, which is equivalent to between 15 and 20% of the diet should supply enough. Twenty per cent of defatted wheat germ in vitamin B_4 low basal rations for chicks protects them from the deficiency.

The poor response obtained with a level of 0.25 gm. of dried pork brain per day shows, as was pointed out above, that the 'goat milk factor' is different from the limiting factor for growth of rats on mineralized winter cows' milk

In spite of the fact that no clear cut vitamin G (B_2 anti-pellagra factor) deficiency has been obtained in rats, it was considered advisable to show definitely that the 'grass juice factor' could not be related to vitamin B_2. This was done by feeding a purified liver extract concentrate which was active in curing blacktongue in dogs and pellagra-like symptoms in chickens. Only a very slight response was obtained.

Since McHenry ('35) found some effect of choline upon the growth of young rats fed on a purified diet, the possibility of choline being active was also considered. Fletcher, Best and Solandt ('35) have reported that both yeast and brain tissue are rich in choline, but neither gave good growth responses as supplements to winter milk.

The definite response obtained with lawn clippings or the juice expressed from grass established the superiority of this material as a source of the factor. The grass was cut fresh every morning from the lawns of the university grounds. It consisted chiefly of Kentucky blue grass. Juice was prepared by pressing out a portion of this freshly cut grass with a screw press. The expressed juice was then clarified by centrifuging for 15 minutes. Each rat received 3 cc. of this cell free

juice per day. Growth curves from some typical experiments are presented in figure 1. Rats receiving this supplement grew as well as any rats on mineralized summer milk. These data are typical of a number of experiments. However, we have had several failures with lawn grass, but these negative results were always obtained when the grass was cut during dry periods when the plants were making very little growth. This suggests that rapidly growing succulent materials are the most potent. Further evidence concerning this problem was

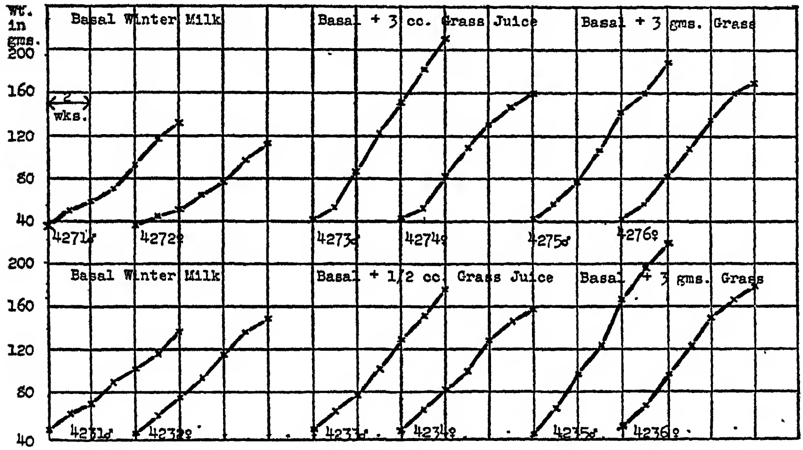

Fig. 1 Showing the effect on the growth of rats of grass and grass juice as supplements to winter milk.

obtained by the use of dried oat grass prepared by the American Butter Company.[5] Sample no. 180 was cut when the oats were very young (18 days old) and contained 40% protein on the dry basis. Sample no. 223 was cut 12 days later and contained only 30% protein. Both samples had been obtained from the same field, and were dried at 160°F. under identical conditions. Sample no. 180 at a level of 0.6 gm. per day, which supplied somewhat more dry matter than 3 gm. of fresh grass, gave very excellent growth. Sample no. 223 cut at a later

[5] We wish to thank Mr. C. F. Schnabel and Mr. Meryl Bowman of the American Butter Company for making this material available to us.

stage, showed distinctly less activity. The fact that these dried samples, especially sample no. 180, gave good growth responses indicates that this factor will withstand drying when carried out under proper conditions. Oats seedlings grown in a greenhouse failed to give as satisfactory results. Thus, the environmental conditions undoubtedly have a profound effect upon the amount of certain nutritive factors found in the plant. Similarly we have found several batches of alfalfa to give poor results. Here, however, enough juice

TABLE 3

Record of milk consumption by the rats on the winter milk and the winter milk supplemented with grass and grass juice

	MALES			FEMALES		
	Gain in body weight in 4 weeks[1]	Milk intake for 4 weeks	Intake of milk solids per gram increase in body weight	Gain in body weight in 4 weeks[1]	Milk intake for 4 weeks	Intake of milk solids per gram increase in body weight
	gm.	*cc.*	*gm.*	*gm.*	*cc.*	*gm.*
Litter 4230						
Control	64	1061	2.41	73	1335	2.66
½ cc. grass juice	98	1454	2.15	77	1613	3.04
3 gm. grass	125	2126	2.47	81	1709	3.06
Litter 4270						
Control	74	1063	2.28	64	940	2.13
3 cc. grass juice	122	1853	2.20	78	1429	2.66
3 gm. grass	112	1563	2.03	86	1699	2.86

[1] The 4 weeks included the third, fourth, fifth and sixth weeks of the experiment.

was prepared at one time to last 6 weeks, so that the active material might have been lost during storage in spite of the fact that KCN was added, and it was kept in an ice box.

The only other materials which showed a potency comparable to that obtained with the leafy plant materials were rice gran and liver extract.[6] Rice bran fed at a level of 0.2 gm. per day gave a surprisingly good response. Liver extract at the same level produced good growth and a higher level, 0.5 gm. per day, gave no better response. However, when a

[6] We are indebted to Dr. David Klein, Wilson Laboratories, Chicago, for the samples of liver extract used.

different batch of the liver extract was tested very little increase in growth resulted. Much more work is necessary before we can make definite conclusions about the distribution of this factor in materials other than rapidly growing young plants.

Consumption records were kept during the last 4 weeks of several of the experiments in which potent grass and grass juice were used, and the results are given in table 3. From these records it can be seen that the males, which were growing faster due to the addition of grass juice or grass, ate correspondingly greater quantities of milk than the controls receiving no grass. Thus, the ratio of food intake to gain in weight remains practically constant. When females receive grass in addition to winter milk, their food intake rises markedly, and, although there is a definite increase in body weight over that of the controls, this increase is not great enough to be proportional to the increase in intake of milk solids. Thus this factor stimulates growth, which in turn increases the food requirement. There is no indication that it increases the efficiency of utilization of the ingested nutrients.

DISCUSSION

The data presented above show that fresh or carefully dried green plant tissues contain a substance (or substances) which is the limiting factor for growth of rats on mineralized winter milk. The activity of the fresh plant tissues may be demonstrated by use of the expressed juice. The daily dose of juice necessary to produce optimum growth is 0.1 gm. on the dry weight basis. The samples of liver extract and rice bran tested showed some potency.

It will be noted that various other supplements produced slight increases in growth over the controls. For example, yeast produced an increase of 0.49 gm./day. This might be interpreted as meaning that yeast contains a small amount of the factor, but it would not be a safe conclusion to make since the difference is not greater than that due to individual variations observed between some of the rats; further there

were not sufficient numbers of animals used to allow statistical treatment of the data.

In view of the fact that grass is most potent when it is cut during the most rapidly growing stage, it was thought possible that plant growth stimulants, the auxins, which have been isolated from urine, might be involved in the growth of these animals. Hence, one of these, heteroauxin (β indolylacetic acid)[7] was tried as a supplement to the basal milk, but no conclusive results were obtained.

von Wendt ('35), in Finland, has published work which indicates that the seasonal variation in nutritive quality of milk is of importance in human nutrition in territories where milk and milk products are the chief articles of diet. He has also reported rat and mouse feeding experiments which demonstrated the nutritive difference between winter and summer milk, although he makes no mention of supplementing these milks with iron, copper and manganese, so that deficiencies of these elements probably complicated his work.

We should also like to emphasize the importance of this problem of winter versus summer pasture milk in relation to child nutrition; to the evaporated and condensed milk industry and to the production of powdered milk. Further what relation does the 'grass juice factor' sustain to the well-known stimulation to milk production observed when cows on winter rations are turned to green pasture, and is its preservation of distinct moment in the health of the cow?

Henry and Kon ('36), of England, have reported recently that they could demonstrate no difference in the growth of rats on summer and winter milk. In their work they used the paired feeding method, limiting the intake of the rats on summer milk to the level consumed by those on winter milk. A consideration of table 2 would lead one to expect that no difference would be obtained using the paired feeding method, since the growth obtained is proportional to intake in the case of the males, where the most striking growth difference is noted.

[7] This sample was kindly supplied by Dr. K. V. Thimann (Harvard University).

Another factor which we consider to be of prime importance is the special care which must be taken to prevent the young rats from obtaining stores of the factor. Kon took no special precautions in the preparation of his animals.

SUMMARY

1. A series of supplements was fed to rats receiving mineralized winter milk ad libitum. Growth was stimulated by daily supplements of 3 gm. fresh grass, 3 cc. grass juice, 0.6 gm. dried oat grass, 0.2 gm. rice bran, or 0.2 gm. liver extract.

2. The known essential food factors, such as vitamins A, D, C, B, B_2, B_4 and B_6, as well as flavin, choline, 'goat milk factor,' and 'alcohol-ether precipitate factor,' have been eliminated from consideration as being the active principles, by the inferior growth responses produced by daily doses of 2 drops of cod liver oil, 1 cc. orange juice, 0.5 gm. brewers' yeast, 0.25 gm. dried brain, and 1.0 gm. defatted wheat germ.

3. Increased growth of rats on mineralized winter milk receiving a grass, or grass juice, supplement over that of controls receiving no supplement is accompanied by an increase in milk consumption.

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UNDERNUTRITION, STARVATION AND PHAGOCYTOSIS [1]

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THREE FIGURES

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Although a considerable literature exists in regard to many aspects of the problem of undernutrition and starvation, not one investigation could be found in which the process of phagocytosis was investigated under these conditions. The only paper dealing with immunological properties under the condition of starvation was that by Zilva ('19), who kept guinea pigs from 10 to 28 weeks on a restricted mixed diet and studied the serum in regard to agglutinin and amboceptor reactions. In these experiments no differences between the titers of the control and experimental animals was observed, which may be due 1) to the small series of animals employed, 2) to the fact that the sera were pooled. The influence of undernutrition and starvation on phagocytosis was made the subject of this investigation 1) because it is in general assumed that the nutritional state of an individual has a great influence on the course of infectious diseases, but no scientific investigation seems to exist to justify this claim on the basis of the immunological properties of the blood; 2) because a study of the effects of losses of body weight on phagocytosis seems essential for a correct interpretation of the results obtained in the preceding studies of Gellhorn and Dunn ('37). These authors showed that vitamin A deficiency in the diet

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diminishes the phagocytosis promoting properties of the serum. Since vitamin A-deficiency is associated with loss in body weight it is important to know whether the alterations in phagocytic index are due to the loss in body weight or to the absence of the specific influence of vitamin A.

METHOD

The experiments were carried out on 127 rats during 1935 and 1936. The control rats were fed with Dickinson's dog food (mixed grains and lettuce). The experimental rats were divided into two groups, the first comprising rats subjected to acute starvation, whereas the second served in chronic undernutrition. Food was withheld completely in the acute experiment. In the chronic experiment the rats obtained the normal food given to the control rats, but in restricted amounts. Ordinarily food was given only every second or third day, depending on the degree of weight loss observed. At the end of each experiment the animals were killed and the phagocytosis promoting power of the sera was compared with that of the controls. The material which was ingested was starch and Hamburger's method was used in a modified form. Concerning all the details, compare the paper of Gellhorn and Dunn ('37).

RESULTS

In the acute starvation experiments rats weighing 200 to 250 gm. were used. They were given no food, but water was given freely. At the end of 6 to 8 days' starvation the animals were killed. Nine experiments were carried out on fifty-eight starving rats, and eighteen controls. Of the fifty-eight rats fifteen, equaling 25.9%, died of pneumonia or other infectious diseases. The results obtained with the rest are indicated in figure 1.

It was obvious from this figure that under the influence of acute starvation the phagocytic index of the serum of the starved animals was quite different from that of the controls in a considerable number of cases. The results, however,

were not uniform, insofar as eleven animals showed an increased phagocytic index, whereas twenty-one showed a decrease in phagocytic index, the remaining number showing a phagocytic power similar to that of the controls.

This result seems to be best explained on the basis that during acute starvation the animals were easily susceptible to various infectious diseases, as is brought out by the high

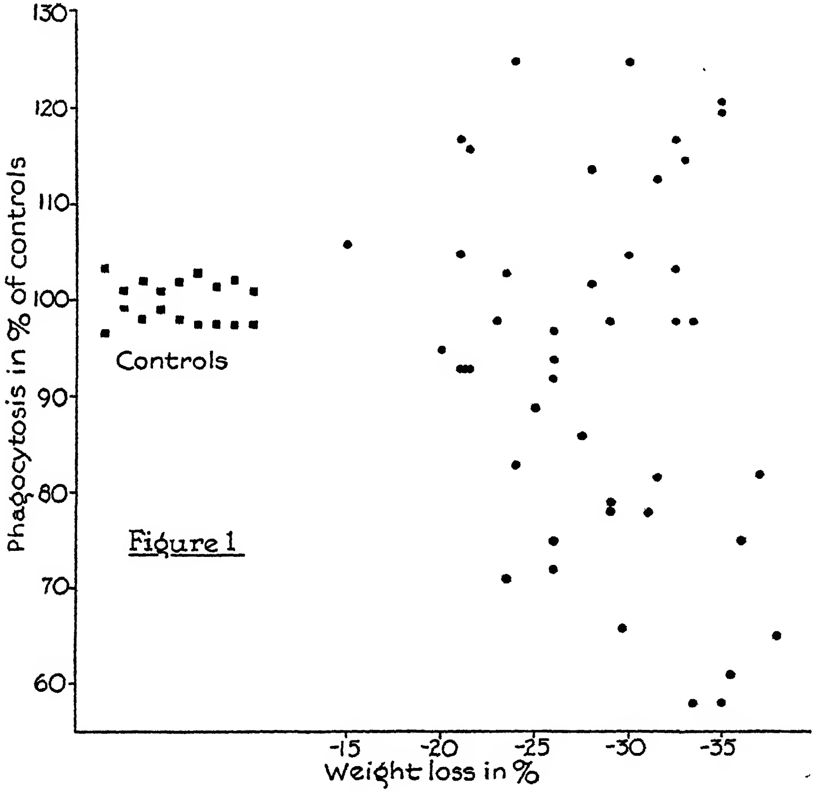

Fig. 1 The influence of acute starvation (6 to 8 days) on the phagocytosis promoting properties of the serum. Normal rat leucocytes obtained by intraperitoneal injection of saponin-Ringer were incubated in a buffered starch-phosphate-Ringer solution containing control and experimental serum, respectively. Squares = controls. Dots = experimental sera. Each dot or square represents one experimental animal. Abscissa: Weight loss in percentage of original weight. Ordinate: Phagocytic index in percentage of phagocytosis obtained in the presence of control serum.

mortality. Under such conditions the phagocytic index may rise. In other cases the phagocytic index is lowered, which may be due to an alteration or exhaustion of the production of opsonins during the starvation process so that the typical response to an infectious process, i.e., an increase in the phagocytic index, will not occur in acute starvation. It is, however, possible that even in the absence of any infectious process the phagocytic index is lowered in starvation. This latter interpretation is most probable in view of the fact that with increasing loss in body weight there is an increased number of cases which show a loss in phagocytic index. Furthermore, the greatest decrease in phagocytic index is observed in cases where the loss of body weight was greatest.

As careful histological studies of the pathological processes which may have been going on in these animals were not undertaken, this explanation is only a tentative one. In order to establish the relationship between starvation and phagocytosis on a firm physiologic basis, it was thought desirable to keep the animals on a restricted diet which may lead to a sufficient loss in body weight, but which is not severe enough on the animal to lower its general resistance, as was the case in the acute starvation experiments reported above. For this reason chronic experiments with undernutrition were carried out in two groups, the first dealing with growing rats, the second with adult rats.

In the first group (fig. 2), six rats, weighing between 90 and 120 gm., were fed every second or third day with our standard diet (see above), so that in the course of 26 days no increase in body weight occurred, whereas the growth in the two controls which were fed every day was considerable. At the end of this period the animals which were apparently in perfect health were killed and the phagocytic index of the serum was determined. The results in table 1 are conclusive. They show that undernutrition which inhibits growth during a period of 4 weeks does not interfere at all with the phagocytosis promoting properties of the serum.

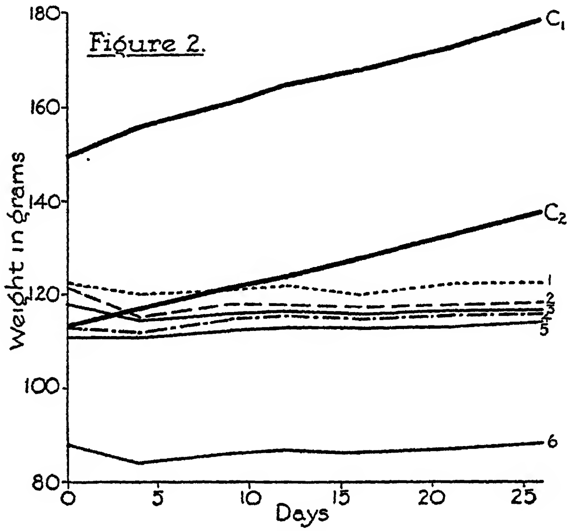

Fig. 2 Weight curves of two control rats (C_1 and C_2) which were fed daily, and six experimental animals which were fed every second or third day with the same standard diet.

TABLE 1

The influence of inhibition of growth on the phagocytosis promoting properties of the serum

SERA	I NUMBER OF LEUCOCYTES WHICH CONTAIN STARCH	II TOTAL NUMBER OF LEUCOCYTES COUNTED	PHAGOCYTIC INDEX I/II	PHAGOCYTOSIS IN PER CENT OF CONTROL
			% %	
Control 1	260	495	52.6 } 51.2	100
Control 2	275	550	50.1 }	100
No. 1[1]	143	293	48.7	95.4
No. 2	86	186	46.2	90.5
No. 3	100	200	50.0	97.7
No. 4	105	200	52.5	102.5
No. 5	95	190	50.0	97.7
No. 6	95	185	50.5	98.7

[1] Nos. 1 to 6, experimental animals fed only every 2 to 3 days so that growth was inhibited.

A last group of experiments was carried out with forty-three rats weighing 200 to 250 gm. Ten rats were kept as controls, the remaining thirty-three were used as experimental animals in an experiment on the effects of undernutrition. Similarly, as in the preceding group of experiments, the experimental animals were fed every second or third day, whereas the controls were fed daily. In five experiments,

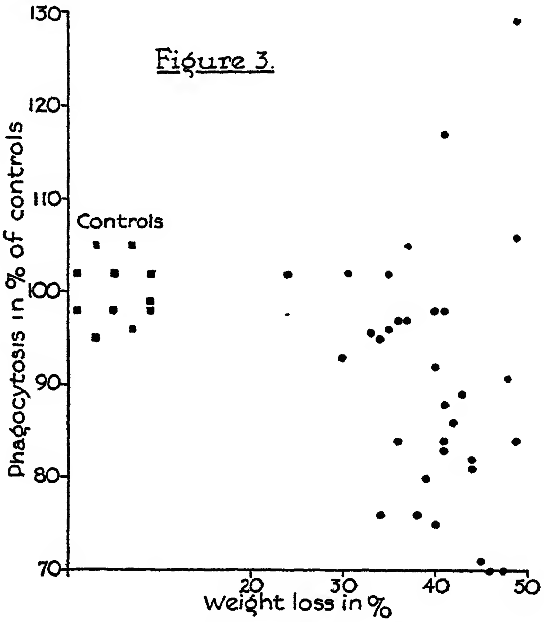

Fig. 3 The influence of chronic undernutrition on the phagocytosis promoting power of the serum of adult rats. The experimental animals (dots) were fed only every second or third day with the standard diet. The duration of the experiments (each with two control animals) varied between 25 and 42 days.

reproduced in figure 3, the duration of the period of undernutrition varied between 25 and 42 days. The loss in body weight varied between 24 and 49%. The animals were killed at the end of the experimental period and were apparently in a good state of health with no outward signs of any infectious processes. The results indicate that with the exception of two rats,[2] which showed an increased phagocytic index,

[2] This experiment was carried out during a severe heat wave in July, 1936.

the phagocytic index of the rats was either within normal limits or, and this was the case in 18 or 55% of the rats, decreased. The decrease in phagocytic index seems to be a function of the loss in body weight, the threshold being at approximately 38% weight loss. Figure 3 shows that the phagocytic index was considerably decreased in sixteen, or 76%, of the rats who showed a loss in body weight of 38% or more.

These experiments show that in the absence of acute infections, undernutrition leading to considerable loss in body weight interfered with the maintenance of a normal phagocytic index of the serum. It gives us a means of evaluating the results previously published by Gellhorn and Dunn. In these investigations it was found that under conditions of vitamin A deficiency a decrease in phagocytic index occurs. There was, however, no parallelism between the decrease in phagocytic index and loss of body weight. Moreover, the experiments reported in this paper show that the phagocytic index is lowered only when a loss of about 35 or more per cent of body weight has occurred. Among the experiments reported by Gellhorn and Dunn only two animals which were subjected to lack of vitamin A for several weeks showed a loss in body weight between 30 and 35%. Neither of these two cases showed a decrease in phagocytic index, which proves conclusively that the results obtained in our former paper were independent of the loss in body weight incurred and due to the absence of vitamin A in the food.

In an attempt to interpret the data presented in this paper, two sets of observations must be considered. The first concerns the serum proteins under starvation and undernutrition. Torbert ('35) showed that the total serum protein decreased slightly under starvation. The differences seem, however, not to be significant, at least as far as the effect of acute starvation for 6 or 7 days is concerned. When he pooled the sera of four control and four fasting rats he found a total protein concentration of 6.9% and 6.95%, respectively. The quotient $\frac{\text{albumin}}{\text{globulin}}$ had, however, decreased from 0.84 to 0.40, indicating even an increase in the globulin fraction. In view of

the fact that phagocytosis is much greater in globulin than in albumin solutions (Kanai, '23; Mudd, McCutcheon and Lucké, '34) the alterations occurring in the serum proteins cannot be the cause of the decrease in phagocytosis during starvation.

The second group of observations concerns the changes in glands with internal secretion under the influence of starvation and undernutrition. It is known from the work of Jackson ('16 and '25), Chang ('25), Rabinovitch ('29), and more recently by Trendelenburg ('34), that in acute starvation and chronic undernutrition the histological picture of the thyroid gland is altered considerably. The decrease in the height of the epithelium and the absence of mitosis seems to indicate a diminished physiological activity. Similar changes, to a lesser extent, are found in the parathyroid glands (Jackson, loc. cit). Since it is known from the work of Asher ('24), Abe ('25), Fleischmann ('27) and others that the phagocytosis promoting properties of the serum are decreased after thyroidectomy and increased after transplantation of a thyroid or administration of thyroxin it seems not unlikely that the results obtained in this study are due, at least in part, to a decreased secretory activity of the thyroid. Concerning the influence of other organs with internal secretion on phagocytosis little is known (compare the review of Mudd, McCutcheon and Lucké). It may, however, be mentioned that the parathyroid may play an important role since histological evidence of decreased physiological activity has been reported by Jackson, and we have found in extensive unpublished experiments that parathormone considerably increases the phagocytosis of starch in vitro.

SUMMARY

Experiments on the effect of acute starvation and chronic undernutrition were carried out in rats and the influence of these conditions on the phagocytosis promoting power of the serum was investigated. It was found that acute starvation produces a definite tendency toward a progressive decrease in phagocytic index in correlation with loss in weight. There were, however, a number of animals with an increase in

phagocytic index probably due to the occurrence of acute infections from which we lost many animals.

In experiments with chronic undernutrition in which the animals were fed only every second or third day during a period varying between 25 and 42 days, it was found that when the loss in body weight was greater than about 38% a considerable decrease in phagocytic index occurred.

Experiments carried out on growing rats, in which the standard diet was fed every second or third day so that no growth occurred during the period of 4 weeks, did not show any change in phagocytic index.

In view of the fact that chronic undernutrition lowers the phagocytic index only when the loss of body weight is about 38% or more the results obtained in a preceding paper showing that during a prolonged period of lack of vitamin A in the food the phagocytic index is decreased, must be considered to be due to the lack of vitamin A and not to loss in body weight.

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THE ROLE OF MANGANESE AND CERTAIN OTHER TRACE ELEMENTS IN THE PREVENTION OF PEROSIS

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Perosis (Titus, '32) is an anatomical deformity of the leg bones of young chickens, turkeys, pheasants, grouse and quail. It is recognized as a disorder entirely distinct from rickets inasmuch as calcification is normal. This deformity was first described by Hunter and Funk ('30) and by Payne ('30). The symptoms generally found are gross enlargement of the tibial-metatarsal joint, twisting or bending of the distal end of the tibia and of the proximal end of the metatarsus, and slipping of the gastrocnemius tendon from its condyles. The latter symptom causes complete crippling in the affected leg and, if both legs are so affected, is closely followed usually by death. The literature dealing with perosis has shown that excess calcium and phosphorus in the diet tend to cause it and that certain feedstuffs, notably those derived mainly from the pericarp of rice, oats and wheat, possess a preventive action. Little is actually known, however, of the actual cause or nature of this deformity.

In a previous paper (Wilgus, Norris and Heuser, '37), the relative effectiveness of different sources of calcium and phosphorus in aggravating the incidence of perosis was studied. Chemically pure calcium carbonate, calcium hydroxide and calcium chloride as well as mono-, di- and tri-calcium phosphate and mono-sodium phosphate were found to be equally

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causative. On the other hand, a technical grade of mono-calcium phosphate was markedly preventive in effect. It was evident that impurities in this phosphate possessed a marked ability to prevent perosis. The purpose of this paper is to report the identification of these impurities and a study of their function in bone formation in the chick.

EXPERIMENTAL PROCEDURE

The experimental procedure was the same as that previously used (Wilgus et al., '37). Day-old cross-bred cockerel chicks (Rhode Island Red × Barred Plymouth Rock) were confined on wire floors in electrically-heated battery brooders for a period of 6 weeks. Individual weighings and notations of deformities were made at weekly intervals. The notations were given a numerical rating so that the severity of any lot could be expressed as the percentage of the maximum severity possible. In the previous paper, it was shown that practically all the individuals in a lot were afflicted when the average severity for that group was between 25 and 30% and that the highest severity noted for any group was 55%.

The chicks had constant access to a basal diet of the following composition:

Ground yellow corn	63.50
Dehydrated alfalfa	5.00
Dried skim milk	20.00
Menhaden fish meal	10.00
Salt (iodized)	0.50
Reenforced cod liver oil	0.25
Refined cottonseed oil	0.75
Total	100.00

This diet contained on the average 20% of protein, 0.95% of calcium and 0.80% of phosphorus. The cod liver oil contained 270 U.S.P. units of vitamin D per gram. The same ingredients were used in all the experiments reported here with one exception as noted later. Mineral supplementation was made by direct addition.

RESULTS AND DISCUSSION

In order to concentrate or isolate if possible the impurity in the technical mono-calcium phosphate previously found to have a preventive effect on the development of perosis, the phosphate was brought partially into solution in cold distilled water, treated with an excess of ammonium hydroxide, and kept at the boiling point for several hours. The precipitated material, essentially tri-calcium phosphate, was removed, washed and ignited to remove the adsorbed ammonia. The filtrate and washing, containing ammonium phosphate, were combined and condensed. These two fractions were added to the basal diet and compared in effectiveness with the original material. One lot of chicks received the basal diet only, a second lot received the basal diet plus the technical mono-calcium phosphate, a third lot received the basal diet plus the tri-calcium phosphate fraction and a fourth lot received the basal diet plus the ammonium phosphate fraction.

The technical mono-calcium phosphate did not prove to be quite as effective in overcoming perosis as it had been originally nor was the lot on the basal diet as severely affected as usual. In spite of this the results showed that the preventive factor was concentrated in the tri-calcium phosphate fraction as there was a marked reduction in the incidence and severity of perosis in the lot of chicks which received this fraction. The ammonium phosphate fraction, on the other hand, showed no observable effect on the incidence of perosis.

This procedure was repeated in a second experiment using the same basal diet as that fed in the first experiment except for the substitution of white fish meal for menhaden fish meal. The basal diet caused more perosis in this experiment. The results obtained were even more striking than those of the first experiment and completely confirmed the previous conclusions.

In a further attempt to obtain clues as to the nature of the factor which prevents perosis an experiment, based on the work of Graham, Pettit, Sykes and Howell ('34) who reported that the perosis-preventive fraction of wheat germ is easily

extracted with cold water after adjustment to the pH of 3.0, was conducted. This extraction procedure was therefore applied to wheat germ which had been extracted first with ether and hot alcohol. The extract from 10% of wheat germ was compared in effectiveness with an equivalent amount of the original germ. In order to determine whether the effect was due to an inorganic constituent, the char from wheat germ was also fed on an equivalent basis. In this experiment, 2.87% of steamed bone meal was added to the basal diet in order to make it more severely perosis-producing.

The results showed that, while the wheat germ reduced the severity of perosis, the extract was much more effective, thus substantiating the findings of Graham et al. ('34) that wheat germ extract prevented perosis. The char was as effective in this respect as the extract, thereby indicating the inorganic nature of the preventive factor.

Spectrographic analysis of the technical mono-calcium phosphate showed that a small amount of manganese and a trace of iron were the principal impurities present in the technical mono-calcium phosphate. These were found to be concentrated by ammonia precipitation in the tri-calcium phosphate fraction. The ammonium phosphate fraction showed only traces of calcium, sodium and possibly aluminum.

The routine qualitative analyses of the technical mono-calcium phosphate and of the tri-calcium phosphate from it indicated the presence of aluminum as well as of manganese and iron. It appeared possible, therefore, that one or all three of these elements might be responsible for the preventive properties found in this material.

The wheat germ, wheat germ extract and the wheat germ char were also examined spectrographically and found to contain considerable manganese as well as magnesium, zinc, sodium, potassium, calcium and phosphorus. Again, the occurrence of manganese appeared to be highly suggestive of its possible importance.

Further evidence of the importance of manganese was found in the fact that the spectrographic examination of the ash

of the hocks of normal and afflicted birds showed the presence of this element in the normal bones and its absence in the deformed ones.

On the basis of such strong circumstantial evidence, the possible effect of the direct addition of manganese to the basal diet was determined in experiment 1. Since iron and aluminum were also present in the technical mono-calcium phosphate, salts of these elements were likewise studied. A zinc salt was included due to the presence of this trace element in the wheat germ fractions. No additions of calcium or of phosphorus were made, since the basal diet produced an intermediate rather than an extreme amount of perosis and hence could be used to determine whether the above salts were preventive or causative.

Since quantitative analyses of the impurities in the original mono-calcium phosphate had not yet been completed, 0.015% of each of these elements was added to the basal diet. The salts used were c.p. manganese chloride ($MnCl_2 \cdot 4H_2O$), ferric citrate ($FeC_6H_5O_7 \cdot 3H_2O$), aluminum chloride ($Al\,Cl_3 \cdot 6H_2O$) and zinc acetate [$Zn(C_2H_3O_2)_2 \cdot 3H_2O$]. The essential data are given in the first part of table 1. Each lot contained fifteen cross-bred cockerel chicks.

As explained previously, the quantity of perosis on the basal diet used in this experiment was considerably above that of the previous experiments, possibly due to the change in fish meals. However, the original mono-calcium phosphate prevented most of the perosis. The manganese salt was not only equally as effective as the impure phosphate but also stimulated growth. The aluminum, iron and zinc salts all had a slight preventive action and a beneficial effect on growth.

The results were so promising that these salts were fed again at the same level to lots 3, 4, 5 and 6 in experiment 2. Each lot contained sixteen cross-bred cockerel chicks. The only change in ingredients in the basal diet consisted in reverting to the use of menhaden fish meal in place of white fish meal.

The results are given in the second part of table 1. They show that manganese chloride was effective in preventing nearly all the symptoms of perosis and that the aluminum chloride and zinc acetate salts were moderately preventive. The ferric citrate, however, actually increased the severity

TABLE 1

The effect of manganese, aluminum, iron and zinc salts

LOT	AMOUNT OF SUPPLEMENT	Ca.	P	AVERAGE WEIGHT AT 6 WEEKS	PEROSIS	
					Cases	Severity
	Experiment 1					
		%	%	gm.	%	%
1	None (basal diet)	1.08	0.83	467	93	35
2	1.6% tech. $CaH_4(PO_4)_2 \cdot H_2O$	1.31	1.23	479	36	7
3	0.015% Mn	1.08	0.83	533	29	7
4	0.015% Al	1.08	0.83	527	43	25
5	0.015% Fe	1.08	0.83	497	77	17
6	0.015% Zn	1.08	0.83	492	72	17
	Experiment 2					
1	None (basal diet)	0.97	0.78	487	87	21
2	1.6% tech. $CaH_4(PO_4)_2 \cdot H_2O$	1.21	1.18	455	40	7
3	0.015% Mn	0.97	0.78	501	27	6
4	0.015% Al	0.97	0.78	511	40	16
5	0.015% Fe	0.97	0.78	421	94	40
6	0.015% Zn	0.97	0.78	469	27	11
7	1.69% c.p. $CaH_4(PO_4)_2 \cdot H_2O$	1.21	1.17	442	94	32
8	1.69% c.p. $CaH_4(PO_4)_2 \cdot H_2O$ + 0.0025% Mn	1.21	1.17	440	25	6
9	1.69% c.p. $CaH_4(PO_4)_2 \cdot H_2O$ + 0.0025% Mn + 0.0025% Al + 0.0025% Fe	1.21	1.17	509	27	6
10	0.0025% Mn	0.97	0.78	503	19	2
11	0.0025% Mn + 0.0025% Al + 0.0025% Fe	0.97	0.78	550	0	0

of perosis in this experiment. This salt was from the same lot as that originally used. The only known change in procedure, other than the change in fish meal, was that the salts were much more intimately mixed in the diet by grinding the whole mixture very finely. Possibly the iron salt interfered with the normal assimilation or utilization of the effective element

by some reaction before the feed was consumed. Such interference has been suggested in plant nutrition between manganese and iron (Young, '35).

In the meantime, quantitative determinations of the amount of manganese in the technical mono-calcium phosphate and in the tri-calcium phosphate precipitated from it had been made by the method of Willard and Greathouse ('17). The former was found to contain 0.170% of manganese and the latter 0.367%. This confirmed the spectrographic analyses showing that the manganese was concentrated in the latter.

With this information, it was possible in a second part of experiment 3 to add the same amount of manganese to the basal diet as was added by the original phosphate, using c.p. mono-calcium phosphate to adjust the calcium and phosphorus levels. The quantity of manganese added was 0.0025%. This amount was fed alone (lot 8) and in conjunction with a similar amount of iron and aluminum to simulate more closely the original material (lot 9). To determine the effectiveness of these supplementations as compared to the much larger amounts fed in the first half of the experiment, a duplicate of each of these groups was conducted without the addition of c.p. mono-calcium phosphate equivalent to the original impure phosphate (lots 10 and 11).

As shown by Wilgus, Norris and Heuser ('37) c.p. mono-calcium phosphate materially increased the severity of perosis. On the other hand, the addition of 0.0025% of manganese to it reduced the severity to the same low level as on the impure technical mono-calcium phosphate (lot 2). The use of a similar amount of iron and aluminum with the manganese was of no additional value. When the calcium content of the diet was reduced from 1.21% to 0.97% and the phosphorus from 1.17% to 0.78%, by eliminating the c.p. mono-calcium phosphate supplement, the manganese alone was nearly preventive, and the mixture of all three salts was entirely preventive. The smaller amount of manganese used in this part of experiment 2 was fully as effective as the larger amount used in the first part.

The data in experiment 2 substantiate the previous results showing the preventive action of manganese, aluminum and zinc. The evidence on iron is conflicting. It therefore seems apparent that the preventive effect of the technical monocalcium phosphate was due mainly to the manganese and possibly to the aluminum present as impurities. The effect of these elements on growth is not so clear in these two experiments, but there is an indication that they may be a limiting factor at times in the basal diet used. The evidence on zinc does not agree with the report of Titus ('32) but it is possible that the small amount fed by him (0.001%) was insufficient to be of appreciable value.

In view of these results it seemed desirable to re-examine results of an experiment previously reported in a preliminary manner (Wilgus, Norris and Heuser, '35), showing the relative effectiveness of various feedstuffs in preventing perosis. The diets used in this experiment were analyzed for manganese, and the amount of this element in each diet was compared to the amount of perosis produced. These feedstuffs were representative of samples, obtained at the Buffalo market early in 1935. The essential data are presented in table 2. Each lot contained twenty-five Barred Rock chicks. The chemical analyses showed that there was a relation between the preventive action of the various diets and their manganese content as in general the diets high in manganese caused the least perosis.

Since manganese appears to play an important role in preventing perosis and possibly in stimulating growth under certain conditions, the amount of it in the individual feedstuffs used in these and in previous experiments was determined and is presented in table 3 together with the calcium and phosphorus analyses. They show that the feedstuffs reported here and elsewhere as possessing a marked preventive action are generally rich in manganese. This is strikingly so in the case of wheat germ and its extract. It is also worthy of note that the basal diets used in producing perosis in this investigation were very low in manganese, containing between 0.0009 and 0.0012%.

The presence of 0.0088% of manganese in the limestone used in previous work (Wilgus, Norris and Heuser, '37) may explain the fact that the highest level of limestone seemed to ameliorate the perosis slightly. The presence of other

TABLE 2

The efficiency of common feedstuffs in preventing perosis in relation to the manganese content of the diet

LOT	AMOUNT OF SUPPLEMENT [1]	Ca	P	Mn	AVERAGE WEIGHT AT 6 WEEKS	PEROSIS	
						Cases	Severity
		%	%	%	gm.	%	%
1	None (basal diet)[2]	1.80	1.20	0.0011	378	100	31
2	10% wheat germ	1.80	1.28	0.0027	496	39	10
3	10% wheat standard middlings	1.81	1.25	0.0025	518	63	23
4	20% wheat standard middlings	1.81	1.31	0.0038	480	10	2
5	30% wheat standard middlings	1.82	1.36	0.0052	498	8	4
6	20% red dog flour	1.80	1.26	0.0025	476	54	13
7	20% wheat bran	1.81	1.39	0.0040	483	48	19
8	20% ground oats	1.80	1.20	0.0017	445	78	24
9	20% hard wheat	1.80	1.21	0.0019	464	78	33
10	10% soybean oil meal	1.81	1.23	0.0014	438	94	37
11	None (basal diet)	1.18	0.79	0.0011	464	80	25
12	—20% corn meal + 20% starch	1.18	0.72	0.0009	414	95	28
13	—5% dehydrated alfalfa + 5% starch	1.08	0.78	0.0010	327	90	33

[1] The supplements were included in the basal diet in place of an equivalent quantity of corn meal, the protein content being kept constant by making suitable adjustments in the casein content and the remainder of the corn meal.

[2] This basal diet was supplemented with sufficient steamed bone meal and pulverized limestone to increase the calcium content to 1.80 and the phosphorus content to 1.20%.

elements, as detected spectrographically, may also have had some effect. Variation in impurities may also partially explain the results of Sherwood ('32) showing that pulverized oyster shell was slightly preventive, those of Insko, Sowell

and Lyons ('34) showing that precipitated calcium carbonate possessed no preventive action, and those of Hunter, Dutcher and Knandel ('31) and of Payne, Hughes and Lienhardt ('32) showing that pure calcium carbonate increased the severity of perosis.

The possible nutritional significance of the trace elements has been suggested at various times and has been supported

TABLE 3

The calcium, phosphorus and manganese content of the feedstuffs used

FEEDSTUFF	Ca	P	Mn
	%	%	%
Corn meal	0.054	0.352	0.0010
Corn meal	0.040	0.356	0.0008
Oats (10)	0.070	0.336	0.0037
Wheat (10)	0.038	0.423	0.0048
Wheat standard middlings (20)	0.115	0.911	0.0144
Red dog flour (10)	0.071	0.647	0.0078
Wheat bran (15)	0.123	1.350	0.0154
Wheat germ (5)	0.076	1.150	0.0163
Wheat germ	0.043	0.936	0.0182
Wheat germ extract	0.048	0.142	0.0158
Dehydrated alfalfa	2.099	0.226	0.0032
Dehydrated alfalfa	1.992	0.229	0.0026
Dried skim milk	1.202	1.025	0.0001
Dried skim milk	1.267	1.017	0.0001
Soybean oil meal (2)	0.218	0.662	0.0036
Menhaden fish meal	5.640	3.695	0.0032
White fish meal	7.031	3.872	0.0021
Limestone	36.920	0.004	0.0088

The numbers in parentheses denote the number of market samples used in obtaining a composite analysis.

by some experimental data. The probable biological importance of manganese, zinc and aluminum, as well as of copper, was noted by Rose ('29) in a review of the literature, but this was based largely on the consistent occurrence of these minerals in animal tissues. Since that date, several specific functions of manganese have been reported. A sufficient supply of this element was found necessary to maintain the desire, or ability, of rats to suckle their young, to prevent

the production of inferior offspring and to prevent testicular degeneration on a low manganese diet (Orent and McCollum, '31), to assure normal growth and ovulation of mice on a milk-iron-copper diet (Kemmerer, Elvehjem and Hart, '31), and to avoid congenital debility in the young of rats on that diet (Daniels and Everson, '35). Drea ('35) concluded from the spectrum analysis of hen eggs and of chick tissues that manganese might be inadequate in the diet and that it is probably of nutritional importance. A deficiency of zinc has been reported by Stirn, Elvehjem and Hart ('35) to result in poor growth, abnormal fur coat and hyperirritability of rats.

The results of the present investigation furnish more specific evidence of the biological significance of manganese, zinc and aluminum. The data indicate that these 'trace' elements may be a limiting factor in the diet for the normal growth of chicks. In addition, they show that these 'trace' elements are concerned with the anatomical structure of the tibiae and fibiae of chicks, thereby ascribing a hitherto unreported role to them.

SUMMARY

1. The presence in the diet of manganese and certain of the other 'trace' elements has been found essential for the prevention of the deformity of the tibiae and metatarsi of chicks, known as perosis. Indications were also obtained that these elements exercised a favorable influence on growth.

2. The addition of 0.0025 to 0.015% of manganese to a diet containing 0.0010% of this element was sufficient to almost entirely prevent perosis.

3. Zinc and aluminum possessed a similar property but were somewhat less effective.

4. A mixture of manganese, aluminum and iron was entirely preventive in the presence of limited amounts of calcium and phosphorus.

5. The perosis-preventing property of certain cereal products was related to their content of manganese.

ACKNOWLEDGMENT

Acknowledgment is hereby made of the cooperation of the Grange League Federation Exchange, Inc., of Ithaca, New York, which made this investigation possible by establishing a temporary investigatorship at Cornell University. The authors are also indebted to Mr. F. M. McNall of the Chemistry Department at Cornell University for his kindness in making the spectrographic examinations.

ADDENDUM

Since this paper was written, Sherwood and Fraps (Poultry Science, 1936, vol. 15, p. 424) have reported that the preventive factor of wheat gray shorts is present in the ash or inorganic fraction, thereby lending support to the above results, and controverting a report made at the same time by Heller and Penquite (loc. cit.) that the preventive factor of rice bran is seemingly not due to its mineral content.

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THE EFFECT OF VITAMIN E DEFICIENCY UPON GROWTH[1]

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SIX GRAPHS

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INTRODUCTION

Evans ('28) reported a study of the effect of vitamin E low diets on growth and vigor. He found that the failure in growth due to vitamin E deficiency characterized only the last phase of growth, "the phase intervening after the attainment of maturity, which is usually viewed as a plateau in the growth curve, though all animals show at this time a slow ascent." A distinct plateauing in weight was noted by about the one hundredth day of life. Evans attributed the stimulus in growth supplied in wheat germ oil to its vitamin E content, as the growth factor was present in the sterol-free, non-saponifiable fraction as was the vitamin E.

Blumberg ('35), in McCollum's laboratory, observed an earlier failure in growth. He showed that in the experimental females, growth was slightly below normal for the first 5 weeks on the diet, slow at the tenth week and very slow or checked by the fourteenth week. Wheat germ oil or its non-saponifiable fraction stimulated growth in these animals.

[1] Aided by grants from the Board of Research and the College of Agriculture of the University of California, the Rockefeller Foundation of New York, and Merck and Company, Inc., of Rahway, New Jersey.

The following materials were generously contributed: Cod liver oil by E. R. Squibb and Sons. Calciferol by Mead Johnson Company, through the courtesy of Dr. C. E. Bills.

Blumberg used a highly purified diet in which the vitamin E was removed by extraction while Evans employed a less pure diet in which vitamin E had been destroyed by the incorporation of a slightly rancid lard in the diet.

Olcott and Mattill ('36) failed to confirm Blumberg's work, although they used a somewhat similar diet. Supplements of wheat germ oil sufficient to insure fertility had little, if any, effect on growth.

Thousands of vitamin E low rats have been reared in this laboratory. These animals, although sterile as shown by the fact that resorption gestations occurred after positive mating, appeared otherwise normal in all respects. This led us to question the need of vitamin E for early growth.

EXPERIMENTAL

The following diets were used:

Diet 392

Casein, commercial, precipitated with HCl	32
Cornstarch (cooked)	40
Salts (McCollum 185)	4
Lard	24

This diet was allowed to stand at room temperature for 2 weeks prior to feeding and was entirely fed during the next week. Supplements were fed six times weekly: brewer's yeast, 1.0 gm., cod liver oil (Squibb's), 2 drops.

Diet 427

Casein, commercial, precipitated with HCl	27
Cornstarch (cooked)	35
Salts (McCollum 185)	4
Lard	22
Cod liver oil (Squibb's)	2
Brewer's yeast	10

The ingredients (except cod liver oil) were allowed to stand at room temperature for 2 weeks and then fed within a week, as with the preceding diet. The cod liver oil was added to the diet on the day of feeding.

Diet 786

Casein, alcohol, ether extracted according to Blumberg	24
Sucrose	72.1
Salts (McCollum 185)	3.9

Supplements were fed six times weekly:

Brewer's yeast extracted according to Blumberg	1.0 gm.
Carotene (S.M.A.) fed in ethyl laurate	80 gamma
Ethyl linoleate (iodine no. 148–149)	60 mg.
Calciferol (in ethyl oleate)	12 international units

The carotene, ethyl linoleate, and calciferol were kept cold, stabilized with hydroquinone, and saturated with carbon dioxide.

Diet 789

Casein, commercial, precipitated with HCl	27
Cornstarch (cooked)	35
Salts (McCollum 185)	4
Wheat germ oil	22
Cod liver oil (Squibb's)	2
Brewer's yeast	10

Diet 791

Casein, commercial precipitated with HCl	27
Cornstarch (cooked)	35
Salts (McCollum 185)	4
Lard	12
Wheat germ oil	10
Cod liver oil (Squibb's)	2
Brewer's yeast	10

This diet was handled in the same manner as was diet 427.

Diet I

Whole wheat	67.5
Casein	15.0
Whole milk powder	10.0
NaCl	1.0
$CaCO_3$	1.5
Milk fat	5.0

METHODS

Littermate sisters (thirty in each group) were placed on diets 786, 392 and 427 at weaning. The opening of the vaginal orifice was noted and daily (except Sunday) smears were made thereafter. Weighings were made at 5-day intervals. A representative number of rats from each group was bred. Resorption gestations resulted in all cases (table 1).

After the vitamin E low nature of all three diets had been established, the individuals of another group of ninety rats were segregated as in the previous experiment and the cycles and growth responses were observed.

However, in this second experiment, growth responses were observed to 160 days on thirty rats on diet 392; twenty-eight rats on diet 427 (two died from respiratory infection); and seventeen rats on diet 786 (ten were given additions to the diet before day 160; three died from respiratory infections) (graph I).

TABLE 1

Maturity and reproductive history of rats on vitamin E low diets

DIET	NUMBER OF RATS	WEIGHT AT 21 DAYS	WEIGHT AT 60 DAYS	AVERAGE OPENING DAY OF VAGINAL ORIFICE	AVERAGE AGE AT FIRST OESTRUS	NUMBER OF RATS WITH 4- TO 7-DAY CYCLES	NUMBER OF RATS BRED	AVERAGE AGE AT BREEDING	AVERAGE DAY APPEARANCE OF ERYTHROCYTE SIGN	PER CENT RESORBING
		gm.	*gm.*		*days*			*days*		
786	30	46	146	47	56	12	16	92	11.4 (limits 6–15)	100
392	30	45	156	49	52	14	21	90	12.2 (limits 7–15)	100
427	30	45	175	40	47	28	25	91	12.3 (limits 7–14)	100

TABLE 2

Vaginal smear observations of vitamin E low rats

DIET	NUMBER OF RATS	WEIGHT AT 21 DAYS	WEIGHT AT 60 DAYS	AVERAGE OPENING DAY OF VAGINAL ORIFICE	AVERAGE AGE AT FIRST OESTRUS	NUMBER OF RATS WITH 4-TO 7-DAY CYCLES
		gm.	*gm.*		*days*	
786	30	48	131	44	57	15
392	30	47	156	45	58	23
427	30	47	173	40	47	19

The growth responses of a part of each group were observed to 210 days (graph II):

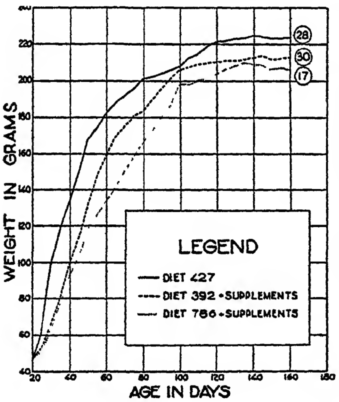
14 rats remained on diet 427
14 rats remained on diet 392
8 rats remained on diet 786

The growth response on diet 427 was somewhat greater than on diets 392 and 786, although their plateaued weights

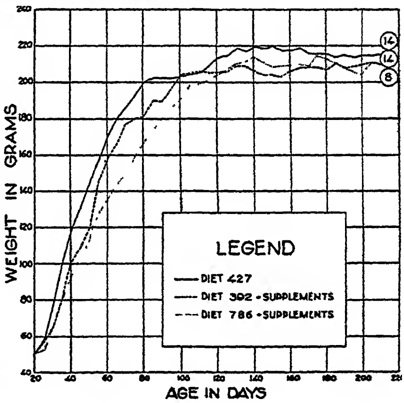
(from day 120) did not differ substantially. This difference may have been due to the fact that the rats did not consume their supplements quantitatively during the early growth period. The rats, even after 210 days of age, were in splendid condition, showing good muscle tone and sleek coats. The growth responses on all three diets differed materially from that observed by Blumberg, whose vitamin E low females reached a maximum average weight of about 160 gm. after 140 days on the diet and declined slowly to about 120 gm.

As can be seen from graphs I and II, the rats on all three vitamin E low diets reached plateaued weights of over 200 gm.

GRAPH I
GROWTH ON VITAMIN E LOW DIETS



GRAPH II
GROWTH ON VITAMIN E LOW DIETS



There are at the present time five rats on diet 427 (which have never been bred) and which average 230 gm. weight at 19 months of age. These animals are partially bald and have an unsteady gait, but have not declined appreciably in weight.

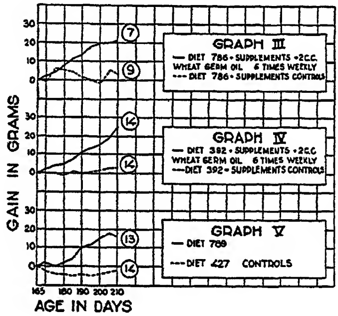
When the rats were 165 days of age, a part of each group was given massive doses of wheat germ oil which had been previously standardized for vitamin E potency and found to be effective in a dose of 500 mg. These groups were as follows:

	Number of rats in group
Diet 786 + supplements + 2 cc. W.G.O. six times weekly	7
Diet 392 + supplements + 2 cc. W.G.O. six times weekly	14
Diet 789 containing 22% W.G.O. fed fresh in porcelain container	13

The rats assigned to diet 789 had been maintained previously on diet 427 and had therefore had no training in eating supplements, hence the wheat germ oil was incorporated in their diet. Diet 789 differs from 427 in that the lard was replaced by wheat germ oil and kept fresh.

The individuals of all groups responded to the addition of wheat germ oil to their diets. The gain was at least 17 gm. over the plateaued weight for each group (graphs III, IV and V).

GRAPHS III, IV & V
THE EFFECT OF WHEAT GERM OIL
UPON LATE GROWTH



	Average increase in weight over plateaued weight after 40 days on diet
	gm.
Diet 786 + supplements + W.G.O.	21
Diet 392 + supplements + W.G.O.	25
Diet 789	17

A pilot experiment was carried out to confirm the presence of the 'late growth' factor in the non-saponifiable fraction of wheat germ oil. Two animals from the 786 group received supplements of 0.5 gm. wheat germ oil six times weekly from the age of 110 days.

Three received, six times weekly, the ethyl esters of the wheat germ oil fatty acids equivalent to 0.5 gm. W.G.O.

Three received, six times weekly, the wheat germ oil non-saponifiable fraction equivalent to 0.5 gm. W.G.O.

Graphs are not given because of the small number of animals employed.

TABLE 3

Growth response of vitamin E low rats reared on diet 786 following the addition to the diet of wheat germ oil, the wheat germ oil non-saponifiable fraction, and the ethyl esters of wheat germ oil fatty acids

	(2)[1] 0.5 GM. W.G.O. SIX TIMES WEEKLY	(3) ETHYL ESTERS FATTY ACIDS EQUIVALENT FOUND IN 0.5 GM. W.G.O. SIX TIMES WEEKLY	(3) NON-SAPONIFIABLE FRACTION FOUND IN 0.5 GM. W.G.O. SIX TIMES WEEKLY	(7) NO FRACTION
Gain from				
Days 110 to 150	20	17	10	10
Days 150 to 190	26	9	51	0
Total gain				
Days 110 to 190	46	26	61	10

[1] Number of animals in group.

Two rats were changed from diet 786 to diet 427 at 95 days.

Gain from days 95 to 110	20
Gain from days 110 to 150	11
Gain from days 150 to 190	2
Total	33

The addition of fat to the diet either in the form of the ethyl esters of the wheat germ oil fatty acids or by changing to the high fat vitamin E low diet 427 seemed to stimulate growth slightly after the controls had ceased growing, but not to as great an extent as did wheat germ oil or its non-saponifiable fraction. As the number of rats on each supplement was small, the results can be considered only as suggestive.

As a result of the observation that wheat germ oil stimulated growth after the vitamin E low rats had plateaued in weight, it seemed advisable to observe the effect of wheat germ oil on the early growth period. Sixty females were segregated into four groups of fifteen each, as nearly uniform as possible, and placed on the following diets at weaning (21 days).

E low diet 427
Stock diet I
Diet 427 + 4 drops wheat germ oil fed directly into the mouth six times weekly
Diet 791

It was hoped that the vitamin E in the wheat germ oil in diet 791 would be destroyed by the rancidity of the lard incorporated in the diet. This, however, did not prove to be the case as the natural antioxidants of the wheat germ oil apparently prevented this oxidation (as can be seen by the results of breeding tests).

Four animals from each group were selected for breeding in such a manner that the average weights of the groups were not affected. The sixteen were bred between the one hundred thirty-fifth and the one hundred forty-first days of life. The results of the breeding were as follows:

TABLE 4

Reproductive performance of rats reared on a vitamin E low diet, a stock diet, and two wheat germ oil containing rations

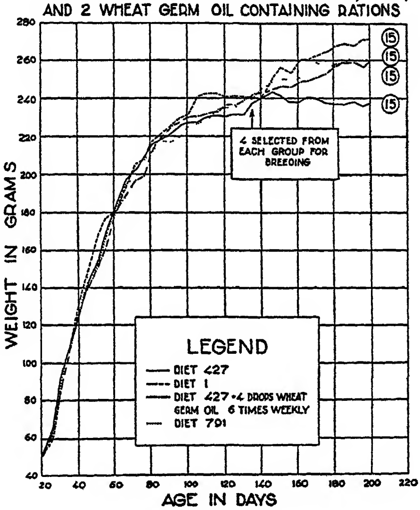
DIET	NUMBER SHOWING ERYTHROCYTE SIGN	NUMBER OF RESORPTIONS	LITTERS	AVERAGE NUMBER OF YOUNG PER LITTER	AVERAGE WEIGHT IN GRAMS
Diet 427	4	4	0	...	...
Diet I	4	0	4	8.3	6.0
Diet 427 + 4 drops W.G.O. six times weekly	4	0	4	7.2	5.8
Diet 791	4	0	4	5.5	5.9

All animals bred on diet 427 resorbed. The litters of the rats receiving wheat germ oil were somewhat smaller than on diet I, but were of average weight.

The rats in the several groups grew at about the same rate until 145 days, as can be seen from graph VI. The weights averaged between 243 and 250 gm. at this point. The animals on the vitamin E low diet had definitely reached their maximum weight; declined slightly and remained at a plateaued weight of about 237 gm. for the remainder of the experimental period (to 200 days). The rats receiving wheat germ oil either in the diet or as a supplement were increasing slightly in weight, averaging from 5 to 10 gm. less than those receiving the stock diet I.

It can be seen from the foregoing experiments that the failure of growth in vitamin E deficiency is a phenomenon of growth after the fourth month of life and that earlier growth is normal; nevertheless it is apparent that in all long continued growth experiments a source of vitamin E should be included in the diet for it is apparent that lack of E will otherwise play a definite contributory role in growth delinquency.

GRAPH VI
COMPARISON OF GROWTH ON VITAMIN E LOW, STOCK, AND 2 WHEAT GERM OIL CONTAINING RATIONS



SUMMARY

1. Three vitamin E low diets were tested for their efficacy in producing the sterility typical of vitamin E deficiency. The vitamin E in one of the rations was removed by extraction (diet 786) and in the other two by the presence of slightly rancid lard in the diet (diets 427 and 392). All three diets proved to be vitamin E free as evidenced by typical resorption gestations.

2. Daily vaginal smears were made on the rats on these diets. The animals on the low fat diet 786 and the high fat diet 392 with supplements exhibited more irregularities in ovulation frequency than did their sisters on the high fat

diet 427 in which the yeast and cod liver oil were incorporated in the diet.

3. The growth of female rats on the three vitamin E low diets was observed. The animals on diet 427 grew more rapidly at the onset, but after 120 days of age the plateaued weights of the three groups were about the same.

4. The individuals in all groups responded to the addition of wheat germ oil to their diets after a plateaued weight had been reached.

5. The growth stimulating factor appeared to be in the nonsaponifiable fraction of the oil, rather than in the fatty acid fraction of the oil.

6. Female rats reared from weaning on a vitamin E low diet (427), stock diet I, and a wheat germ oil containing diet (791), and diet 427 supplemented with wheat germ oil, grew at the same rate until about 145 days, at which time the animals on the vitamin E low diet plateaued in weight while those on the stock diet and the wheat germ oil containing diets continued to grow.

7. The rats reared on diet 427 were sterile; the rats on diet I, diet 791, and diet 427 + wheat germ oil were fertile.

8. The defective growth in experiments involving vitamin E deficiency occurs only after the fourth month of life. Wheat germ oil stimulates growth after a plateaued weight has been reached on a vitamin E low diet.

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BASAL METABOLISM OF RATS IN RELATION TO OLD AGE AND EXERCISE DURING OLD AGE

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THREE FIGURES

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As part of its investigation of energy metabolism in different animal species at different ages, with the special object of illuminating human physiology, the Nutrition Laboratory has studied short-lived mammals such as the rat, which is considered to live about thirty times as fast as man. Relatively few laboratory rats are maintained throughout their entire lives, and previous measurements of the metabolism of rats of advanced age are practically confined to those recorded by Benedict and MacLeod ('29) and Horst, Mendel and Benedict ('34 a).[1] During the past 3 years we have been able to maintain a considerable number of rats under constant laboratory conditions until they die from natural causes. This has made possible a cooperative research by Columbia University and the Nutrition Laboratory on the metabolism of some of these rats during old age.

The average age of the elderly rats studied by Benedict and MacLeod corresponds approximately to a human age of 60 years. In the present investigation a number of measurements of basal energy metabolism were made upon rats whose ages corresponded to ages of 30 to 50 years in the human

[1] After our research was completed, Prof. J. E. Davis, of The University of Chicago, courteously gave us the opportunity of reading a manuscript written by him reporting his study of sleeping rats, with the privilege of referring thereto in our paper.

life cycle, but much the larger number were made on rats of more advanced ages, several having lived to ages corresponding to 80 to 90 years in human life. In view of the necessity for extreme condensation in this presentation of our data, we shall here deal chiefly with the observations at well advanced ages, 750 or 800 days and over, as compared with observations upon the same population at ages of 450 or 500 to 700 or 750 days. In other words, we seek to note the changes, if any, in basal metabolism in old age as compared with the basal metabolism in middle age.

Our second main problem is, to what extent does a known amount of enforced muscular exercise affect the basal metabolism of rats already middle-aged when exercise begins. In this study we were interested, not in the immediate after-effect of exercise, but in the effect, if any, upon the basal metabolism measured a sufficient time after the exercise to ensure the absence of any immediate after-effect. An incidental object of these experiments was to note the effect upon the length of life of thus beginning in middle age a rigorous program of exercise in individuals previously sedentary. Rubner ('08) laid down a general thesis that an individual of a given species metabolizes a relatively constant number of calories of energy in the course of a life cycle. From this standpoint increased energy metabolism induced by muscular exercise might tend to shorten life. Although the number of rats studied in this part of our investigation was too small to furnish a crucial test of Rubner's thesis, it was thought that the data obtained might throw some light upon it and should in any case afford an indication as to whether strenuous muscular exercise begun and enforced relatively late in life results in a permanent increase of the basal metabolism unaffected by the immediate after-effects of work.

One hundred and thirteen middle-aged or old rats, varying in individual cases from 352 to 789 days of age at the beginning of their respective series of observations, and from 423 to 1113 days at the end, were included in this investigation. Of these rats, forty-two were males and seventy-one females,

including nine males and six females that were subjected to enforced exercise.

All the rats were descendants of Osborne-Mendel albino stock and may be considered a homogeneous group for the purpose of the present investigation.

Technic of metabolism measurements

The metabolism was studied in a multiple four-chamber respiration apparatus, which provided for individual measurements on four rats simultaneously and kymograph records of the activity (Benedict, '30). The animals were all subjected to a 24-hour fasting period. They were measured at 28°C., and kept for 48 hours at 28°C. preceding each respiration experiment. At other times the animals lived in rooms that were almost constantly within the temperature range of 22° to 26°C. Except when in the respiration chamber they lived without bedding in wire mesh cages with liberal ventilation and diffused light, water always available, and food available at all times save during the fasting periods preceding the metabolism measurements.

It was impossible to control activity. Careful observation and the kymograph records aided in selecting those periods of complete muscular repose. The animals were not usually asleep, and hence this factor, known to depress the metabolism of humans, did not as a rule enter. To favor repose, an electric light shone over the chamber, with the thought that this nocturnal animal might become quieter under brilliant illumination. Pieces of cardboard were placed between the glass chambers to prevent the rats from seeing and perhaps exciting each other. It was found that the rat often sought privacy by putting his head into a small hood provided in the chamber.

Length of experimental period. The degree of repose which a waking animal can be expected to maintain is usually inversely proportional to the length of the period of measurement. Hence our technic for measuring the basal metabolism aimed to set the period of measurement short enough for

minimal activity but not too short for experimental accuracy of measurement. Our measurements were based upon the length of time required by the rat to absorb a given amount of oxygen, approximately 180 cc. After a dozen or more measurements of such length were made throughout the day, the longest periods (ca. 35 to 40 minutes) on each day were selected as being indicative of minimal activity and hence in our judgment truly representative of the basal metabolism. In only rare instances was the basal metabolism value as thus selected on any day based upon only one experimental period. Usually it represented the average of at least the two longest periods and ordinarily more.

NON-EXERCISED RATS

Total heat production, referred to age, of equal-weight groups

To study the effect of age upon basal metabolism, the age factor should theoretically be isolated as completely as possible from other factors, especially weight. Inasmuch as a large mass of data was accumulated during this investigation, a number of metabolism measurements are available on rats weighing much the same but of widely different ages, from the middle to the end of the normal life cycle. Thus in figure 1 has been plotted, with reference to age, the average 24-hour basal heat production of each of the non-exercised female rats that at the time of observation was within the weight range of 210 to 230 gm. This is approximately the normal average range in weight of the adult female rats in our particular colony, which have always been bred from individuals of only average size and, therefore, now have an average weight smaller than that of laboratory rats in many other colonies. In figure 1 the hollow circles represent the average values obtained upon those rats that reached an age of 800 days or over before the end of the series of observations included in these average values. The solid dots represent those rats that were observed only at ages below 800 days.

The age of 800 days, here somewhat arbitrarily used as a dividing line between middle-aged and old rats, corresponds

(according to the generally accepted approximate ratio of 30:1) to about 65 years of age in human life. All the rats in the middle-aged group included in figure 1 were over the age of 400 days, corresponding to about 33 years in human life, so that the younger of these two age groups is free from any cases of the intensification of metabolism attributable to youth and physical growth. In the ordinary sense of the terms, physical development had been fully completed or the 'prime' fully attained before the beginning of any of the metabolism measurements here recorded.

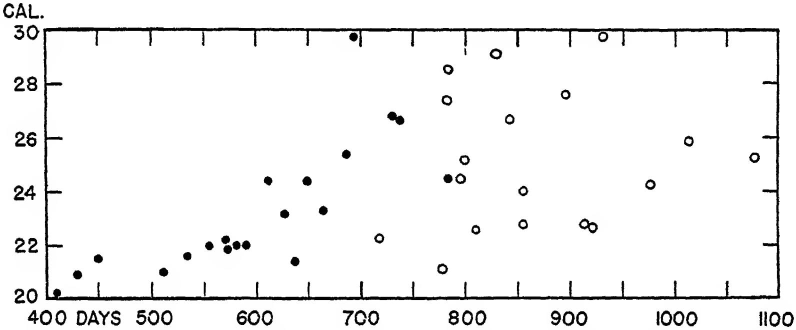

Fig. 1 Total 24-hour basal heat production referred to age, of non-exercised female rats of equal weight (210 to 230 gm.). The hollow circles represent the average values for those rats that reached an age of 800 days or over by the end of the series of observations on them, the solid dots those for rats observed only under 800 days old. This chart shows the slightly higher basal metabolism of the old age group as compared with the middle aged group of essentially the same body weight.

This same type of analysis was applied to our data for females weighing from 190 to 209 gm., and for males weighing 230 to 250 gm., the charts for which are here omitted for economy of space.

These comparisons of basal metabolism in normal adult rats that differed widely in age but were of nearly equal weight show a slight increase in the basal metabolism of the rat in old age, at least under the conditions obtaining in the colony from which the rats here used (as well as those previously studied by Benedict and MacLeod) were drawn. This is

probably the nearest approach that has been made to the isolation of the age factor as such, particularly the separation of age-change from weight-change. Inasmuch as weight was here approximately the same (and surface computed from weight also necessarily the same) for the groups of different age, the somewhat higher average basal metabolism measured in the old age group is equally distinct as an experimental finding whether stated on a per capita, a per kilogram or a per square meter basis.

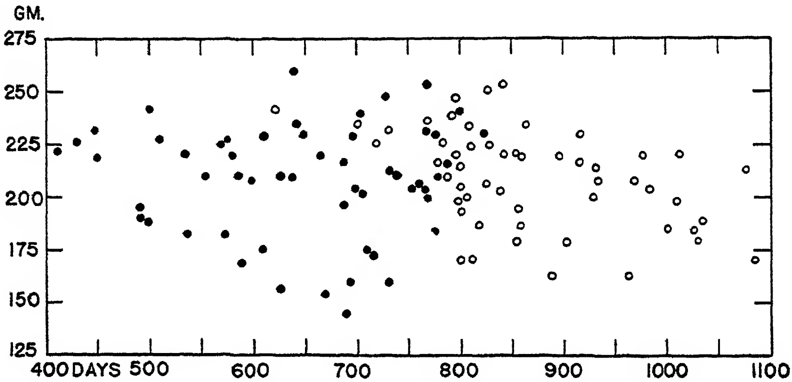

Fig. 2 Body weight referred to age, of non-exercised female rats. The hollow circles and the solid dots have the same significance as in figure 1. The data are not limited to rats in the weight group of 210 to 230 gm. but include all the comparable non-exercised female rats studied.

Trend of changes in body weight, body temperature and basal metabolism with advancing age

The values for the average body weights and the average total heat production of our non-exercised females have been plotted with reference to advancing age in figures 2 and 3, respectively. The data for males are fewer and are omitted for economy of space.

Body weight. Our males showed a distinctly downward trend in weight with advancing age, the average for this population being as follows: between 350 and 550 days, 321 ± 8 gm.; between 550 and 750 days, 289 ± 5 gm.; 800 days and over, 267 ± 8 gm. The weights of the females showed no

uniform dominant trend. Thus one series of population measurements gave between 400 and 600 days an average weight of 208 ± 3 gm.; between 600 and 800 days, 207 ± 3 gm.; 800 days and over, 206 ± 2 gm. On the other hand, in a series in which the same individual females were observed in middle life and after 800 days of age, the average weight was found to decrease about 10%, i.e., from 224 to 202 gm.

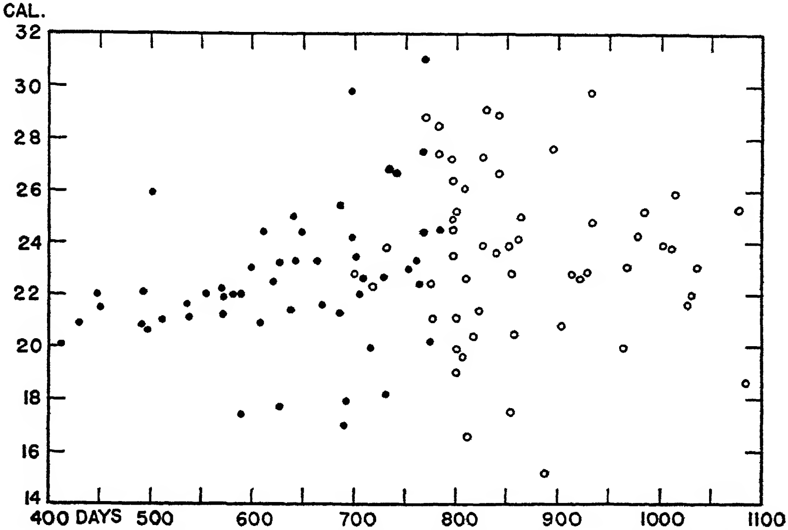

Fig. 3 Total 24-hour basal heat production referred to age, of non-exercised female rats. The hollow circles and the solid dots have the same significance as in figure 1. The data are not limited to rats in the weight group of 210 to 230 gm. but include all the comparable non-exercised female rats studied.

As the females of this rat colony live about 5 or 6% longer than the males of the same hereditary and nutritional history, we have here allowed 30 to 50 days more of chronological age to females than males in setting the zones of physiological age for comparison, i.e., if we divide the males at 750 days we divide the females at 780 or at 800 days of age.

Body temperature. The rectal temperatures, taken just before or just after the measurements of basal metabolism but excluding those when the rats were in a moribund condition, showed a tendency to decline in old age. This was

usually apparent only after the age of 800 days. Among these oldest rats, however, the decrease of temperature may ultimately amount to as much as 2° to 3°C.

Total heat production referred to age, irrespective of changes in body weight

For both sexes, an outstanding feature of our measurements as a whole is the close approach to constancy of average per capita heat production throughout the latter part of the natural life cycle (fig. 3). The average total basal metabolism computed to 24 hours was close to 26 calories for the males averaging 250 to 270 gm., and close to 22 calories for the females averaging 210 to 230 gm. The data afforded by this study are fundamentally concordant with the findings of one of us (Benedict, '35; Benedict and Meyer, '32), that the total 24-hour heat production of women 78 years of age and over, and presumably of elderly women in general, appears to be close to 1000 calories, irrespective of weight or age.

As our data are more numerous and conclusive with respect to females than to males in both parts of this investigation, the limited space here available is given chiefly to the observations upon the females, and even these can be reported only in condensed form. To this end, the original measurements were first reduced to a single monthly average basal metabolism datum for each rat. These data for those rats that were observed over sufficiently long and sufficiently advanced age ranges were then averaged in three groups for the age ranges 420 to 599, 600 to 779, and 780 days and over, respectively. The average total calories per 24 hours for these three age groups, with the numbers of monthly average data in each, were as shown in table 1. Here the downward trend in basal heat production per capita is clearly established, is measured in large enough numbers of comparable cases to justify expression of precision (as P.E., the classical probable error), and is shown to be both statistically significant and very small.

Basal heat production per 24 hours per square meter of body surface

In view of the tendency to a downward trend of body weight in old age, it may be deemed necessary for purposes of comparison to express these data also with reference to size. For such comparative purposes the conventional computation to the basis of surface area will probably best serve the convenience of the reader. The surface area was computed from the weight by means of the formula

$$S = 9.1 \times w^{2/3}$$

From our data it appears that for these populations believed to be rather strictly comparable there is an exceedingly

TABLE 1

Average total 24-hour basal metabolism of non-exercised female rats

AGE RANGE	NUMBER OF CASES	CALORIES (MEAN WITH P.E.[1])
Group I, 420 to 599 days	68	22.09 ± 0.15
Group II, 600 to 779 days	83	21.64 ± 0.17
Group III, 780 days and over	74	20.85 ± 0.18

[1] This is the classical probable error of the mean.

slight upward trend with age in the heat production as thus computed from measurements made on the waking rat under as nearly basal conditions as were found experimentally obtainable. This confirms the finding of Benedict and MacLeod, but in our judgment it is truer to the scientific significance of our measurements to emphasize their relative constancy than to emphasize the very slightly upward trend of the results when thus referred to size. That the upward trend is real and that these observations, therefore, confirm those of Benedict and MacLeod is indicated further by the fact that when the total heat production of rats of markedly different ages but of essentially the same weights are compared, somewhat higher figures are found for old age than for middle or upper-middle age. This latter comparison has the advantage of dealing with a pure age difference uncomplicated by

significant differences in weight, but it has also the disadvantage that, since there is a downward trend in body weight with age, the group of old rats having the same weight as the middle-aged group had had greater weights in their middle age and should, therefore, more properly be regarded as essentially larger individuals. Hence the upward trend of the basal metabolism per unit of weight or of surface area in old age must be regarded as being a slight one. This upward trend with rats and the downward trend with humans are so slight that the angle of divergence between them is probably too small to be of much, if any, scientific significance. The fact that a horizontal line falls within this angle tends to give the slight divergence an exceedingly exaggerated sound when one puts it in words and says that the trend in one case is upward and in the other case downward. It would be well, therefore, to avoid this form of statement.

Comparison of same individuals studied in middle age and old age

In most of those cases in which the same individuals were studied before and after 750 or 800 days of age, the body weight was lower at the older ages and likewise the total heat production. In a series of twenty females studied at both ages the average body weight before 800 days was 224 gm.; the per capita heat production, 23.6 calories; the heat production per kilogram, 104 calories; and per square meter, 696 calories. These same twenty individuals when over 800 days old showed an average weight of 202 gm., a per capita heat production of 21.7 calories, a heat production per kilogram of 107 calories, and per square meter of 693 calories.

Thus when the same individuals are followed from middle age to old age the per capita heat production is found to decrease. But the body weight decreases in somewhat greater proportion than the total or per capita heat production, so that when the total calories are divided by body weight or by surface area as computed from weight, the fact of the per capita decline of heat production is obscured. Although the

individual rat actually lowers its total heat production in its old age, this heat production is made to appear higher in old age if expressed per unit of weight. Furthermore if the total heat production is divided by the surface area as computed from the weight, this rate of heat production per square meter is unchanged in old age in this particular series of rats studied at both ages and shows a very slight increase in our series as a whole, as also in the experience of Benedict and MacLeod.

Our general average finding of heat production per square meter in forty-four normal female rats (regardless of the oestrous cycle), which were repeatedly observed above the age of 400 days and before showing senile breakdown or a moribund condition, was 699 calories. The average of the sixteen males measured at 800 days and over was 710 calories. Another series of measurements upon females showed an average of 699 calories at ages under 800 days and 703 calories per square meter per 24 hours at ages over 800 days. Hence for comparison with other animal species ordinarily measured when awake we suggest that the heat production per square meter per 24 hours of the middle aged to elderly but not seriously senile rat, in as nearly a truly basal condition as is practically attainable with rats that are fully awake, be considered as approximating very closely 700 calories.

EXERCISED RATS

Nine male rats and six female rats, ranging in age from 393 to 623 days at the beginning of these experiments and in body weight from 180 to 362 gm., were subjected to enforced exercise after a preliminary period in which their basal metabolism without enforced exercise was thoroughly established. For comparison with these exercised rats, seven male rats and ten female rats served as controls, being in all but one instance litter mates of the exercised rats. These controls received the same diets as their litter mate exercised rats, were kept at the same environmental temperature, and in every way possible led the same life except that their activity was spontaneous and not enforced and was doubtless very

slight as compared with that of their exercised brothers and sisters. All were of the same colony as those used in the old age study.

The cages in which the rats were exercised have already been described (Reed et al., '30). The rats were made to run for 2 minutes in these cages and then rested for 1 minute. On the first day of exercise the rats were in the cages for 15 minutes. The period of exercise was increased approximately 15 minutes daily, until finally the rats were in these revolving cages for a maximum period of 6 hours daily. As the cage revolved twenty-eight times per minute and the circumference of the cage was 96.5 cm., the maximum distance each rat ran in 6 hours amounted to 6485 meters. This is at the rate of 1081 meters per hour.

By making the sides of the cage smooth and (based upon the experience of the late Prof. M. S. Pembrey) by lining the interior of the rim of the wheel with corrugated paper, it was made nearly impossible for the animal to hang on with the feet and be carried over or to slide, and hence the animal was compelled, for the most part, to run.

When the rats were to be studied in the respiration chamber, they were put into the glass house at 28°C. as soon as their exercise on a given day ended (3 P.M.). Here they remained without enforced exercise for exactly 42 hours prior to the respiration experiment. For 24 hours before the respiration experiment they were without food. Except for these rest periods preceding and during the measurement of the basal metabolism, each exercised rat was forced to run in the revolving cage daily except Sundays and legal holidays. The conditions of environmental temperature, the handling, and the feeding of these exercised rats and their controls were the same as for the non-exercised old age group of rats. In an earlier investigation with rats in the Yale colony (Horst, Mendel and Benedict, '34 b) the same type of exercise cage was employed, and it was found that the immediate aftereffect of running 5486 meters in 6½ hours had disappeared within 40 hours. Hence we concluded that the metabolism

measurements on our exercised rats made 42 hours after the enforced exercise ceased would not be complicated by the immediate after-effect of the exercise and, therefore, would serve to indicate whether enforced exercise permanently alters the basal metabolism of the rat in middle and old age.

Observations

Six of the nine male rats died in the second month of this enforced exercise; the seventh died at the end of the fourth month with a rapid premortal loss of about one-third of his body weight; the eighth died in the sixth month, with a loss of only 17 gm.; the last died in the seventh month of enforced exercise with a loss of about 77 gm.

The females adjusted themselves to the enforced exercise very much better than did the males. Of the six exercised females, five died in the third, fourth, sixth, sixth and tenth months of enforced exercise, respectively, and one was still thriving (in spite of this very considerable amount of enforced exercise) at the end of 13 months, at which time it became necessary to discontinue the work with the revolving cages. The ages attained by the exercised females averaged fully 2 months greater than the ages of their immediate controls or than the general experience of the Columbia laboratory with rats similarly fed. The exercise, therefore, appears to have prolonged the lives of the females, but the number is too small to be more than indicative.

Basal metabolism of rats exercised in the latter part of the life cycle. As briefly indicated above, this part of our research is concerned not with the transitory, immediate after-effect of exercise, but with the question whether regularly enforced strenuous exercise in the latter half of life has any permanent effect upon the body's basal rate of energy metabolism. A change in either direction is easily conceivable. One might reason that exercise would increase the tone of the muscles, so that the basal metabolism would be raised to a higher level, or that regularly recurrent periods of exercise would serve to purge the musculature of whatever it is that

causes an ill-defined restlessness in sedentary middle age, and that after such a purge or release the resting periods would show a more effective relaxation and thus a lowered basal metabolism. The latter possibility, although perhaps less obvious than the former, is probably in better accord with the most expert present-day view of the significance of exercise to the habitually sedentary individual, and of the significance of the ability to relax in rest periods.

Our data show a distinct trend to lower basal metabolism in animals exercised as above described. This is more clearly seen and more certainly demonstrated in the females than in the males, probably because the males did not succeed in adapting themselves to the enforced exercise and usually died in the early stage of the experimental series.

If exercise results in a more rapid lowering of body weight than that ordinarily accompanying old age in the non-exercised rat, this would in itself be expected to lower the per capita heat production but not the heat production per unit of weight or per unit of surface area as calculated from weight. In general, our exercised rats individually showed the gradual decrease of body weight usual in this colony with advancing age, and then, as a terminal symptom, a rapid premortal loss of weight which in some cases was and in other cases was not apparent upon a regular weighing day, in which latter case it was included in the group average. Hence the weight records of these exercised rats are of somewhat uneven value for use in the interpretation of the directly observed respiratory data. But it remains a significant fact that even when the basal metabolism is referred to weight, or to the surface area as calculated from the weight, lower basal rates are found in the exercised than in the non-exercised elderly rats.

GENERAL DISCUSSION OF FINDINGS

That exercise (whether through some such purging or release of chronic, useless, middle age tension as suggested above, or in whatever way) has the net effect of preventing the slight rise of basal metabolism which our rats tend to

show with advancing age and even results in a lowering of basal metabolism is a finding of considerable significance to the interpretation of our observations upon metabolism in old age and the integration of these with the observations of Benedict and Meyer ('32) upon elderly women. The dominant feature of the picture presented by our extension of the study of the basal metabolism of the rat into and typically throughout the latter half of the life cycle is the relative constancy of the basal metabolism found per individual of either sex. For the experimental population of this rat colony the average basal metabolism per individual (per capita) closely approximates 26 calories for males of 250 to 270 gm. and 22 calories for females of 210 to 230 gm. For both sexes the average basal value approximates closely 100 calories per kilogram and 700 calories per square meter of body surface. These averages per kilogram and per square meter are closely similar to those found in such previous investigations as have been conducted under comparable conditions and may, therefore, be regarded as confirming such previous findings and extending them to a more advanced range of age.

Naturally, in view of the fact that a day in the life of a rat is equivalent to a month in the life of a man or a woman, the terminal illness, or period of physiological breakdown which precedes the rat's death, is usually only of a very few days' duration. Often, though not always, it is accompanied by or manifested in a moribund condition as described by Benedict and MacLeod ('29). In any case the basal metabolism measurements recorded in this paper have not included animals in this condition of physiological breakdown. Our measurements have, however, usually extended at intervals of about 10 days or 2 weeks until within a week or two of natural death, and of the animals which were brought for shorter or longer periods within the scope of this study, autopsies after natural death were obtained on the very great majority (96 out of 113), through the courtesy of Doctors Jobling, Wilens and Sproul of the Columbia University Department of Pathology. These autopsied rats showed a wide variety of

lesions and no predominating cause of death. The situation seems fairly analogous to that of people dying in old age.

As already intimated, the aspect of our voluminous data which we believe to be of greatest scientific significance is the almost horizontal trend of the curve of the total 24-hour basal metabolism with advancing age. When submitted to closer analysis, the data show a slightly downward trend in the total 24-hour basal rate of heat production per individual. Simultaneously, there occurs a decrease in the body weight of most of these individuals, and in the average of any considerable group of them. On the average, the decline in body weight is slightly greater than the decline in per capita heat production. Hence, if the heat production be divided by the weight, the slightly downward trend of the original measurements is given a slightly upward trend on the basis of calories per kilogram, and also on the basis of calories per square meter when the body surface is computed from the body weight. This latter confirms the finding of Benedict and MacLeod. Although the upward trend of the data thus figured to the basis of surface area is so small that to dwell upon it is likely to give an exaggerated impression, yet inasmuch as this finding of Benedict and MacLeod was unexpected, we have been interested both to confirm it and to consider possible explanations. Of primary significance are the facts that there is not an actual upward trend in the total 24-hour basal heat production of the majority of individuals as they grow older, and that there is a greater downward trend in body weight than in heat production. The decrease in body weight represents, in all probability, loss of fat or water, with lesser drafts upon protein. Although it is commonly considered that water and fat are metabolically inert, other researches of the Nutrition Laboratory (Benedict and Lee, '36) clearly show that fat is certainly an energy-demanding factor in the body complex. The normal loss of body material in old age, which may be considered adventitious water and fat, tends obviously to diminish the divisor by which the actual total heat production is divided when computed to the basis of weight or surface area.

As briefly noted above, our animals tended to show a slight decrease in body temperature with advancing age. One's first thought in this connection obviously is that with the lower temperature, the rate of oxidation in the cells might be expected to be lowered. But as our expressions of basal metabolism are in terms of heat output, we should not overlook the further consideration that if diminished body temperature means diminished efficiency of temperature regulation, this diminished ability of the body to conserve its heat would tend to a greater output of heat, for the body of the rat was about 10°C. above that of the environment in which its metabolism was measured and about 13° to 15°C. above that of the environment in which it lived between times. Hence, although the observations were made within the zone of thermic neutrality, there is nevertheless the possibility that the heat production of these rats may have been adventitiously increased to a slight extent by the diminution of their efficiency of physical regulation of body temperature, as indicated by the lower body temperatures found in many of the older animals, and in the general average of them.

That the environmental temperature at which these animals were maintained and their basal metabolism measured was within the zone of thermic neutrality, and also that the season of year was not an appreciably disturbing influence in this investigation, have, we believe, been adequately established by our observations.

A further factor in interpreting these results is the question of training. Our rats did not begin their experience in the respiration chamber until after having reached middle age. The well-known lessened adaptability of animals in middle and old age may have made it impossible for these rats to adjust themselves so completely to the new experience as to lose all tenseness and assume the degree of placidity that might have obtained had they been accustomed to the experimental regime earlier in life and at more frequent intervals. Hence it is of distinct interest to observe that the exercised females, having successfully adjusted themselves

(as the males did not) to a considerable amount of regularly enforced activity, were apparently thereby enabled to relax more completely during their rest periods in the respiration chamber.

SUMMARY

Previous investigations of the basal metabolism of the rat have here been extended by observations upon about 100 over 1 year of age. In most cases periodic measurements of the waking basal metabolism at thermic neutrality (28° C.) and after 24 hours of fasting were begun in the period of middle age and continued throughout the latter half of the normal life cycle. Measurements in the moribund state or immediately premortal physiologic breakdown are not included in the data discussed in this paper, but the normal old-age measurements usually continued until within 2 weeks of the natural death of the animal, and necropsies showed no predominance of any one type of lesion or cause of death. Presumably these rats lived out their natural life cycle.

Groups of rats of widely different ages and essentially equal average weight showed a somewhat higher total basal metabolism per 24 hours in old age than in middle age. The trend is clearest in the comparison of the groups that were most numerous and in which the comparison is probably most valid and conclusive. This is probably the closest approach to the isolation of a pure age factor that has yet been accomplished under satisfactory experimental control.

With our rat population as a whole, of rather widely differing weights as well as ages, studied throughout the latter half of the life cycle, we have found a tendency similar to that noted by Benedict and Meyer with elderly women, namely, to a relatively constant basal metabolism for the elderly individual. For these rats this average waking basal heat output per individual per 24 hours is: for males of about 250 to 270 gm., about 26 calories; for females of about 210 to 230 gm., about 22 calories.

Expressed with reference to size, the average becomes, for both sexes, about 100 calories per kilogram, or about 700 calories per square meter, for the basal waking metabolism per 24 hours. These averages may be regarded as sufficiently established for purposes of comparison with other animal species.

Although the relative constancy in total 24-hour basal metabolism is a significant feature of our data, a closer scrutiny reveals that the actual basal heat output per individual decreased slightly with advancing age and that at the same time there was (in the majority of individuals and in the general average) a relatively larger, though also small, decrease in body weight. Hence with rats in advancing age we find a slight decrease of per capita basal metabolism and at the same time a slight increase per unit of weight or per unit of surface area (computed from weight). The latter finding confirms that of Benedict and MacLeod.

The present data show also a tendency to decrease of body temperature in old age. This is usually apparent only after 800 days of age, but in very advanced age may amount to about 2° C.

When middle-aged rats, not previously exercised, were given vigorous enforced exercise daily (except Sundays and the days devoted to the basal metabolism measurement with its preliminary fasting and resting at regulated environmental temperature), the males failed to adjust themselves to such strenuous exercise begun so late in life, lost weight rapidly, and died. The females, on the contrary, were evidently benefited by the exercise, and its permanent effect was shown in a distinct tendency to a lower basal metabolism.

As a possible explanation of the fact that the basal metabolism, as computed per unit of weight or surface area, shows a slightly upward trend with age in the non-exercised but not in the exercised rats, we suggest that the organism is freed by muscular exercise from something in the nature of a middle age restlessness or chronic useless tenseness and so is enabled to relax better in rest periods. The exercised

aging rat thus becomes comparable to the wholesome, co-operative, able-to-relax person who constitutes a satisfactory subject and from whom, therefore, the accepted data for basal metabolism of elderly people are chiefly obtained. The non-exercised rat corresponds to a less-able-to-relax person, whose lowest attainable waking metabolism is a little above the truer basal of the more placid subject. The fact that the aging rats studied by Benedict and MacLeod and by ourselves were (with the exceptions here separately recorded) unexercised, and the further fact that they showed some indications of a diminished efficiency of temperature regulation (not supplemented by clothing, bedding, or warmer rooms as with elderly people) may together explain what would superficially appear to be a slight species difference in the trend of the energy metabolism with advancing age.

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EFFECT OF VITAMIN B DEFICIENCY ON HEAT PRODUCTION OF THE RAT[1]

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INTRODUCTION

The relation of vitamin B(B_1) to tissue oxidation, including the gaseous metabolism of rats in various states of nutrition, was investigated, and the literature to 1926 was critically discussed, by Drummond and Marrian ('26). They concluded that the nutritive failure of rats following a deficiency of vitamin B is virtually identical with that resulting from starvation. Also Lawrow and Matzko ('26) arrived at essentially the same conclusion, from the O_2 consumption of two hens, measured in the initial stage of B avitaminosis.

In a recent report of the effects of vitamin B deficiency on the utilization of food energy and nitrogen by McClure, Voris and Forbes ('34) there was observed a distinctly lower body temperature in the deficient rats, and also a consistently higher carbon to nitrogen ratio in the urine, attributable to the deficiency of vitamin B, and not to prolonged inanition. This difference, however, was not accompanied by a lower heat production in the deficient rats, determined as the balance of food energy not recovered in the urine, feces and body gain.

In order to determine whether extra activity of the deficient rats made their heat production inconsistent with their lower body temperature and higher urinary carbon, this experiment

[1] Authorized for publication on July 23, 1935, as paper no. 696 in the journal series of the Pennsylvania Agricultural Experiment Station.

was repeated, with periodic determinations of the heat production of rats on vitamin B deficient and supplemented diets under conditions of control such as virtually excluded voluntary muscular activity.

The essential features of the initial experiment were repeated, the rats being fed by the paired method, and the utilization of food energy and nitrogen determined from analyses of urine, feces and body gain.

To supplement the previous work, and to follow the progressive change in the utilization of energy and nitrogen during prolonged B deficiency, periodic analyses were made of the urine and feces.

EXPERIMENTAL

Six young male albino rats, 24 to 26 days of age, and weighing from 50 to 60 gm. each, were selected by pairs from the same litter and of approximately equal weight.

The rats were kept in metabolism cages similar to those described by Swift, Kahlenberg, Voris and Forbes ('34). Instead of a crystallizing dish to collect the urine, however, each cage was fitted into an 8-inch glass funnel leading to an Erlenmeyer flask. A thin piece of glass wool in the funnel prevented contamination of the urine. Sulphuric acid was used to protect the urine from loss of nitrogen. The feces were collected on a stainless steel screen fitting into the top of the funnel.

One rat of each pair received a B-deficient and the other a B-supplemented diet. These two diets were made up from three components—*a*) a vitamin B free basal diet of dextrin 64%, casein 18%, Crisco 10%, Osborne and Mendel salt mixture 4%, cellu flour 2%, and cod liver oil 2%; component *b*) vitamin B concentrate, prepared and donated by Parke, Davis & Co., diluted with autoclaved yeast in such measure that 1 gm. contained 10 Sherman units (10 mg.) of the concentrate; and component *c*) autoclaved yeast, prepared by mixing dried brewer's yeast to a thick paste with saturated sodium carbonate solution, and heating for 6 hours in steam, at 120°C. and 18 pounds pressure.

In the preparation of the two dietary treatments the components *a, b* and *c*, were combined to form three rations, which were employed in shifting proportions, as will be explained, to make the B-deficient and the B-supplemented treatments.

The three rations were compounded from *a, b* and *c* as follows: ration I, 90% *a*, and 10% *c*; ration II 90% *a*, 8% *c*, and 2% *b*, containing 1 mg (1 unit) of B concentrate in each 5 gm.; and ration III, 90% *a*, 8% *b*, and 2% *c*, containing 4 mg. (units) of B concentrate in each 5 gm.

These three rations each contained 3.01% nitrogen, 5.80% moisture, and 4628 calories per gram. The components *b* and *c* contained 6.08% nitrogen, 6.93% moisture and 4102 calories per gram.

These rations were essentially the same as fed in the previous experiment except that the vitamin supplements were mixed with the ration, rather than fed separately.

One rat of each pair, hereinafter referred to as the deficient rat, received rations I or II (mostly II) which contained only enough of the vitamin B concentrate to prevent entire loss of appetite. The other rat of each pair, referred to hereafter as the supplemented rat, received ration III throughout the 12 weeks of feeding.

The food intake was kept the same for both rats of a pair, the rat consuming the least food determining the amount alloted to its pair mate. Among 175 refusals of feed, the deficient rats refused six times to one refusal by the supplemented rats.

Beginning with the eighth week for pairs numbered 1 and 2, and the seventh week for pair number 3, 0.5 gm. of component *b* was added to the daily ration of the supplemented rats, and 0.5 gm. of component *c* was added to the daily ration of the deficient rats. This magnified the differential of the vitamin B concentrate, and assured adequate intake of the other factors of the vitamin B complex.

The dates of the collection periods and respiration measurements are given in table 1. Each period was, in effect, a separate metabolism experiment, in which the urine collected

was analyzed for nitrogen and carbon, and the feces were collected and weighed. The accumulated feces were then analyzed for nitrogen and energy at the end of the 12 weeks, and the results applied to the weighed feces of the several periods.

The energy of the urine was obtained from the carbon content of the fresh urine by multiplying by the factor 11.47. This factor was obtained from the previous experiment, and is quite constant for both the supplemented and the deficient rats.

A period began when an animal was put into the respiration chamber, and such quantities of excreta as were passed during the respiration measurement were added to those which followed.

TABLE 1

Schedule of experimentation

PERIOD	PAIR 1			PAIR 2			PAIR 3		
	Started	Ended (respiration)	Age	Started	Ended (respiration)	Age	Started	Ended (respiration)	Age
			days			*days*			*days*
A	Dec. 5	Dec. 12	34	Dec. 6	Dec. 13	33	Dec. 13	Dec. 21	36
B	Dec. 12	Dec. 19	41	Dec. 13	Dec. 20	40			
C	Dec. 19	Jan. 9	62	Dec. 20	Jan. 10	61	Dec. 21	Jan. 11	57
D	Jan. 9	Jan. 23	76	Jan. 10	Jan. 24	75	Jan. 11	Jan. 25	71
E	Jan. 23	Feb. 6	90	Jan. 24	Feb. 7	89	Jan. 25	Feb. 8	85
F	Feb. 6	Feb. 20	104	Feb. 7	Feb. 21	103	Feb. 8	Feb. 22	99
G	Feb. 20	Feb. 26	111	Feb. 21	Feb. 27	110	Feb. 22	Feb. 28	106

The respiration measurements were made in the apparatus described by Forbes, Kriss and Miller ('34) modified by regulation of the temperature of the chamber through that of surrounding air instead of surrounding water. Also, the activity of the rats was controlled by the use of suitably sized bottles as respiration chambers, instead of by confinement in a screen cylinder. Thus the activity of the rats was controlled without subjecting them to discomfort; and the usual inclination of the rats to restlessness was subdued by a bright light, with the result that the heat measurements,

after the first hour, were comparable. The heat production of both rats of a pair was measured at the same time under identical conditions.

At the end of 12 weeks for pairs 1 and 2, and 11 weeks for pair 3, the rats were killed by asphyxiation; the bodies were dried in a vacuum desiccator, and the fat was extracted with ether. The energy contents of the ether extract and of the extraction residue were determined in a bomb calorimeter, and the extraction residue was also analyzed for nitrogen.

RESULTS OF EXPERIMENT

The results, as a whole, of the present experiment, confirm those reported previously, by McClure, Voris and Forbes ('34).

Again there is an uncertainty as to whether vitamin B has a specific effect upon growth. Among thirty-two weekly comparisons the weight of the deficient rats exceeded that of the supplemented rats thirteen times, while the supplemented rats exceeded the deficient rats nineteen times. The comparison of the actual weekly gains gives the advantage to the supplemented rats, by 0.70 gm., but the probable error of this average, which is $\pm$ 0.21, renders doubtful the significance of the slight superiority of the growth of the supplemented rats.

The results of the previous report are also qualitatively confirmed, in that the composition of the bodies, and of the body gains, shows that the supplemented rats utilized the food energy to better advantage than did the deficient rats. The food intake of the rats in the present experiment was much less than in the earlier experiment, and the analysis of the bodies of the rats showed that all had lost fat. The average loss by the deficient rats was 2.03 gm., as compared with a loss of 1.36 gm. by the supplemented rats. The supplemented rats stored, on an average, 16.7 calories in energy as body gain, while the deficient rats lost 0.7 calorie, which is accounted for by their greater loss of fat.

UTILIZATION OF FOOD ENERGY

The digestibility of energy producing nutrients was practically the same for the deficient and the supplemented rats, averaging 90.7% for the former, and 90.6% for the latter. This digestibility diminished from 95% in period A to 88% in period G, the change being continuous and similar for the deficient and the supplemented rats.

The metabolizable energy averaged 84.9% of the energy intake for the deficient rats, and 85.4% for the supplemented rats, the difference being due to the consistently greater energy in the urine of the deficient rats. The same general fall occurred in the metabolizability of the food energy, throughout the experiment, as in digestibility. The metabolizable energy in period A averaged 90.4%, while in period G it averaged 81.0%

The heat loss, measured as the energy of food not recovered in the urine, feces and body gain, was much the same for both the deficient and the supplemented rats. Of 1320 calories intake the deficient rats lost 1118 calories, or 84.6%, as body heat, while the supplemented rats lost 1114 calories, or 84.4%.

UTILIZATION OF FOOD NITROGEN

No difference between the deficient and the supplemented diets as to digestibility of the nitrogen compounds was observed. The average digestibility was 81%, which ranged from 89% in period A to 77% in period G, exhibiting a continuous periodic decrease.

From the digested nitrogen the deficient rats retained an average of 7.3% as body gain, whereas the supplemented rats retained 11.2%.

The ratio of carbon to nitrogen in the urine of the deficient rats was without exception higher than that in the urine of the supplemented rats. This ratio averaged 1.08 for the deficient rats and 0.99 for the supplemented rats.

COMPARATIVE HEAT PRODUCTION

The respiration measurements for the first five periods were made without interruption of the regular feeding habits of the rats. In these periods the daily feed was given 24 hours before the start of the respiration measurements, and the time at which each individual finished his feed, or ceased to eat, was not observed. In periods F and G the feed was removed 15 hours before the start of the respiration measurements.

Some indication of the relative nutritive states of the deficient and supplemented rats at the time of the respiration measurements may be had from the respiratory quotients (table 2). In periods A, B and C the respiratory quotients of the supplemented rats denote a fasting condition and signify that these rats had consumed their allotment of feed more promptly than had the deficient rats. Respiratory quotients were higher in periods D and E.

In periods F and G the respiratory quotients are essentially those of fasting since the feed was withheld from both the deficient and the supplemented rats for 15 hours prior to the respiration measurements.

The hourly heat production of the deficient rats and their supplemented pair mates for the various periods are given in table 2. The heat productions were calculated from the average hourly CO_2, determined over a period of 6 hours, and the total R.Q.

The measurements of the heat production were corrected to represent 100 gm. body weight, in proportion to the two-thirds power of the body weight.

Throughout the early periods, from the first to the eighth weeks, the metabolism of the deficient rats was consistently lower than that of their supplemented pair mates. In period A the average hourly heat production for the deficient rats was 6.2% lower than that of the supplemented rats; in period B it was 8.1% lower; in period C 6.4% lower, and in period D 3.2% lower. There were no exceptions in this respect, and the differences definitely indicate an impaired metabolism in the

TABLE 2

The effect of vitamin B deficiency on the hourly heat production

PAIR NO.	AVERAGE DAILY FOOD INTAKE (DRY MATTER)	VITAMIN B DEFICIENT					VITAMIN B SUPPLEMENTED				
		Vitamin B concentrate per period	Hourly heat as measured	R.Q.	Weight	Hourly heat per 100 gm.	Vitamin B concentrate per period	Hourly heat as measured	R.Q.	Weight	Hourly heat per 100 gm.
					Period A						
	gm.	*mg.*	*cal.*		*gm.*		*mg.*	*cal.*		*gm.*	*cal.*
1	3.2	4	389	0.81	56	573	19	415	0.83	55	615
2	3.4	4	393	0.77	57	571	20	407	0.73	57	591
3	4.6	5	428	0.84	65	568	27	456	0.71	63	621
Average						571					609
					Period B						
1	3.1	5	367	0.79	61	509	18	395	0.75	57	574
2	3.9	4	411	0.74	62	563	23	434	0.74	63	591
Average						536					583
					Period C						
1	3.1	14	390	0.90	69	499	56	386	0.71	60	542
2	3.4	11	334	0.76	60	469	60	382	0.71	66	504
3	3.4	13	399	0.80	69	511	62	411	0.73	67	536
Average						493					527
					Period D						
1	2.7	8	366	0.80	68	471	32	382	0.88	64	513
2	2.5	8	322	0.78	58	462	29	355	0.75	67	463
3	3.1	9	386	0.76	66	509	37	425	0.80	74	519
Average						481					498
					Period E						
1	3.3	8	370	0.77	68	476	69	416	0.97	73	511
2	3.2	8	352	0.83	65	467	68	409	0.96	79	478
3	3.6	10	433	0.83	71	541	71	428	0.83	77	508
Average						495					499
					Period F						
1	3.6	9	386	0.75	68	497	108	431	0.73	68	555
2	2.9	7	361	0.73	59	511	99	378	0.75	70	478
3	3.9	10	478	0.73	71	598	112	487	0.71	77	578
Average						535					537
					Period G						
1	3.4	5	390	0.76	71	488	51	379	0.75	68	488
2	3.0	3	352	0.75	60	495	49	379	0.75	70	479
3	3.9	2	451	0.75	68	581	56	485	0.73	81	558
Average						521					508
Over all average			387	0.78	65	518		412	0.78	68	535
Standard deviation			± 38			± 42		± 34			± 47
Coefficient of variation			9.7			8.2		8.3			8.8

deficient rats which certainly was not the result of inanition—the quantity of feed consumed being the same for both groups, at all times.

However, in periods E, F and G the hourly heat production of the deficient rats was equal to or greater than that of the supplemented rats in six of the nine comparisons, and no differences are evident from the averages. Also, the heat production, which had shown a continuous decline from periods A to D, increased in periods E, F and G, and this increase was more decided for the deficient rats than for the supplemented rats.

This change was coincident with, and was probably the result of the addition of 0.5 gm. of component *b* (autoclaved yeast plus vitamin B concentrate) to the daily ration of the supplemented rats and 0.5 gm. of component *c* (autoclaved yeast unsupplemented) to the daily ration of the deficient rats. While this increased the difference in units of vitamin B concentrate between the deficient and the supplemented rats, the autoclaved yeast may have furnished sufficient vitamin B to bring the deficient rats on a par with the supplemented rats in heat production. However, the deficiency symptoms of depressed appetite and elevated carbon to nitrogen ratio in the urine of the deficient rats persisted throughout the experiment.

The total heat production determined by the body balance method, including all the factors of feeding and cage activity was practically the same for the deficient and supplemented rats, averaging 1116 calories for the 12 weeks. The heat production for an equivalent period under conditions as imposed in the respiration measurements was estimated to be 757 calories for the deficient rats and 804 calories for the supplemented rats. These results imply that the lower heat production of the deficient rats determined under conditions of controlled activity was obscured under the conditions of cage activity.

The general average of the hourly heat production per 100 gm. body weight of the deficient rats was 3.2% lower than that

of the supplemented rats. The odds, calculated by Love's ('24) modification of Student's method are 85 to 1 that this difference is significant. However, to conclude that a prolonged insufficiency of vitamin B produces a continuous reduction in metabolism would be misleading, in view of the equalization of the metabolic rates in the later periods. In this relation, it is apparent that short time experiments may lead to erroneous interpretation of results.

SECOND SERIES OF RESPIRATION MEASUREMENTS

In order to clarify the results obtained in the foregoing experiment a second series of respiration measurements was conducted in which the conclusions of the former work were put to more critical tests.

The paired-feeding and heat measurements excluding voluntary activity were continued. The analyses of urine, feces and bodies were discontinued since observations of McClure, Voris and Forbes ('34) were adequately confirmed in the first series.

In the first series the vitamin supplements were mixed with the ration and although the paired-feeding method assured an equal intake of the heat stable fractions of the vitamin B complex, it did not assure an adequate intake. Consequently, the technic was modified by feeding the yeast supplements separately: 0.75 gm. of autoclaved yeast containing 0.1% vitamin B concentrate (component *b* of the first series) was fed daily to the supplemented rats and the same quantity of autoclaved yeast without concentrate (component *c* of the first series) was given to the deficient rats. The vitamin B-free basal ration was the same as that in the first series.

In order to have the supplemented and deficient animals in a comparable state of nutrition during the time of the respiration measurements the feed offered was curtailed on the previous day to an amount the rats would eat readily. The measurements were begun at least 16 hours after the rats had consumed an equal quantity of feed.

In order to establish or disprove the reality of the reduction in heat production by the vitamin B deficiency, the rats were subjected to reversal of treatment. That is, after two or more measurements of the heat production the deficient rat of a pair was supplemented with vitamin B concentrate while its pair mate was made deficient.

The experimental results of this second series are reported in table 3.

Four comparisons of the respiratory metabolism were made with pair no. 4. Two weeks after the start of the feeding treatment rat no. 8 on the deficient diet had a slightly higher metabolism than the supplemented rat no. 7. Two weeks later the metabolism of rat no. 8 was slightly less than that of rat no. 7. At this point the treatment was reversed, that is, rat no. 7 was made deficient and rat no. 8 was supplemented. The hourly heat of rat no. 7 was 3% lower than that of rat no. 8 after 3 weeks and 5% lower after 4 weeks. The treatment was again reversed but rat no. 7 failed to respond to the curative treatment and the pair was discarded.

Five measurements were made with pair no. 5. Two weeks after the start of the feeding treatment the hourly heat per 100 gm. produced by rat no. 10 (deficient) was 5% lower than that of rat no. 9 (supplemented), and 2 weeks later it was 13% lower. The treatment was reversed. After 3 weeks the metabolism of rat no. 9 was 5% lower than that of rat no. 10 and after 4 weeks it was 7% lower. This pair was put on stock diet until their weights exceeded 100 gm. and returned to the test diets with rat no. 10 deficient and rat no. 9 supplemented. After 10 days the metabolism of rat no. 10 was 3% lower than that of rat no. 9.

Five measurements were made with pair no. 6. Two weeks after the rats were started on the test diets the hourly heat per 100 gm. produced by rat no. 12 (deficient) was 8% less than that produced by rat no. 11 (supplemented). After 4 weeks and 6 weeks the metabolism of the deficient rat was 16% and 12%, respectively, lower than that of the supplemented rat. Three weeks after reversal of treatment the

TABLE 3

The effect of vitamin B deficiency on the hourly heat production

DATE	PAIR NO.	VITAMIN B DEFICIENT					VITAMIN B SUPPLEMENTED				
		Rat no.	R.Q.	Hourly heat as measured	Body weight	Hourly heat per 100 gm.	Rat no.	R.Q.	Hourly heat as measured	Body weight	Hourly heat per 100 gm.
				cal.	*gm.*	*cal.*			*cal.*	*gm.*	*cal.*
11–25–35	4	8	0.74	463	69	593	7	0.77	456	69	584
11– 9–35	4	8	0.76	401	74	490	7	0.71	404	74	494
11–23–35	4	7	0.76	477	85	531	8	0.76	488	84	546
12–30–35	4	7	0.75	451	81	518	8	0.75	478	82	544
11–26–35	5	10	0.73	478	77	568	9	0.74	511	79	597
12–10–35	5	10	0.76	380	76	456	9	0.67	428	74	522
12–31–35	5	9	0.75	442	79	516	10	0.74	517	92	545
1– 9–36	5	9	0.76	399	78	469	10	0.74	476	92	502
1–24–36	5	10	0.73	661	120	585	9	0.76	647	111	602
11–27–35	6	12	0.73	471	81	542	11	0.70	506	79	591
12–11–35	6	12	0.77	364	80	421	11	0.76	410	74	501
12–26–35	6	12	0.75	380	76	456	11	0.81	423	74	517
1–13–36	6	11	0.76	444	87	487	12	0.75	472	97	481
1–21–36	6	11	0.76	438	82	498	12	0.75	482	92	508
11–29–35	7	14	0.76	445	81	512	13	0.73	493	80	570
12–12–35	7	14	0.76	336	74	447	13	0.75	336	66	443
12–24–35	7	14	0.76	407	73	500	13	0.77	370	61	513
1–16–36	8	16	0.72	699	137	565	15	0.74	751	133	619
1–20–36	8	16	0.73	617	130	516	15	0.74	701	131	584
1–27–36	8	15	0.75	676	133	557	16	0.74	627	131	522
Average			0.75			511 ± 46		0.74			539 ± 43

metabolism of rat no. 11 (deficient) was 1% higher than that of rat no. 12 but 1 week later it was 2% lower.

Three measurements were made on pair no. 7. Four weeks after the rats were started on the test diets the hourly metabolism of rat no. 14 (deficient) was 10% less than that of rat no. 13. Respiration measurements 2 weeks and 3 weeks later showed the hourly heat production per 100 gm. to be about the same for these rats. Rat no. 13 persisted in losing weight quite rapidly although rat no. 14 (deficient) maintained a fairly constant weight on the same food intake. Rat no. 14 consistently limited the feed consumption indicative of its deficiency. Because of the apparent abnormality in rat no. 13 the pair was discarded.

In order to observe the effect of the deficiency in larger rats pair no. 8 was introduced. Three weeks after the rats were started on the test diets the metabolism of rat no. 16 (deficient) was 9% lower than that of rat no. 15 and 2 weeks later it was 12% lower. One week after reversal of treatment it was 6% lower.

From the above results there seems to be little reason to doubt that vitamin B deficiency produces a definite reduction in the heat production of rats. The average hourly heat production per 100 gm. was 511 calories for the deficient rats and 539 calories for the supplemented rats. The odds are 3330 to 1 that the two averages are statistically different.

DISCUSSION

The lesion of vitamin B deficiency in rats appears to be localized in the central nervous system as evidenced by the histological observations of Prickett ('34), the interpretation of nervous symptoms by Church ('35) and the studies in tissue respiration by O'Brien and Peters ('35). These last workers have established a definite relationship between crystalline vitamin B and the respiratory behavior of brain tissue, particularly in the reactions of intermediary carbohydrate metabolism.

The reduced energy metabolism and body temperature encountered in vitamin B deficient animals might be attributed to 1) a real interruption of the respiratory processes involved in some step in intermediary metabolism, 2) a reduction in organic and muscular tone resulting from inhibition of nervous control, or 3) a combination of 1 and 2.

The author wishes to acknowledge his appreciation for the provision of facilities and the encouragement by Dr. E. B. Forbes in the course of this work.

SUMMARY

A series of twenty comparisons was made of the heat production of rats on vitamin B(B_1) deficient and vitamin B supplemented diets. The rats were fed by the paired method, and heat measurements were made by the open-train Haldane procedure, at intervals during an experimental period of 12 weeks. The heat production of the B deficient rats was significantly the lower during the first 7 weeks, but in the course of the last 5 weeks the heat measurements representing the two dietary treatments became essentially alike, apparently as a result of an increase in the quantity of autoclaved yeast fed to both groups.

A second series of twenty comparisons, with the technic modified to test critically the results of the first series, definitely established a reduced heat production in the vitamin B deficient rats. Reversal of treatment of paired rats produced reversal in the relative rates of heat production by the deficient and supplemented rats.

Evidence serving to supplement and to confirm the previous findings of McClure, Voris and Forbes is also presented, especially as indicating that with the B supplemented rats a larger proportion of the ration was metabolizable; there was a more favorable body balance of energy; there was less energy and a lower proportion of carbon to nitrogen in the urine; and of the digested nitrogen a larger proportion was retained as body gain.

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A FURTHER CONTRIBUTION TO THE DERIVATION OF FACTORS FOR COMPUTING THE GASEOUS EXCHANGE AND THE HEAT PRODUCTION IN THE METABOLISM OF PROTEINS [1]

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In a recent paper Kriss and Miller ('34) described the derivation of factors for computing the gaseous exchange and the heat production in the metabolism of casein, based largely on complete balances of nitrogen, carbon and energy. The authors found these factors to be considerably different from those originally published by Loewy and Oppenheimer ('11) for casein and for meat protein. In view of the paucity of fundamental data upon which to base the computation of the respiratory products of protein metabolism it was thought desirable to extend the study to the derivation of factors for a variety of proteins.

The present paper is concerned with the derivation of respiration and energy factors for three different animal proteins, namely, heart muscle, casein and gelatin. Casein was included again in this study with a view of determining to what extent, if any, the feeding of the protein as a supplement to a mixed ration, as compared with exclusive protein feeding, will affect the derived factors.

The subjects of this investigation were six male albino rats, each weighing approximately 200 gm. With each of these rats nitrogen, carbon and energy balance studies were conducted while on each of the following daily dietary treatments:

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1) Basal maintenance ration (8 gm., composed of 93.7% calf meal and 6.3% butter fat). 2) Basal ration plus 1.5 gm. dried heart muscle. 3) Basal ration plus 3.0 gm. dried heart muscle. 4) Basal ration plus 1.5 gm. casein. 5) Basal ration plus 3.0 gm. casein. 6) Basal ration plus 1.5 gm. gelatin. 7) Basal ration plus 3.0 gm. gelatin.

The rats received the diet to be tested during a period of not less than 10 days. The first 5 days constituted the preliminary interval of adjustment. This was followed by a 5-day metabolism period during which the urine and feces were collected separately.

The urine and feces of each rat were analyzed for nitrogen, carbon and energy. The diets were similarly analyzed (table 1). From a comparison of the excretory products obtained

TABLE 1

Analysis of proteins fed

SUBSTANCE	MOISTURE	ASH	ETHER EXTRACT	NITROGEN	CARBON	ENERGY
	%	%	%	%	%	*calories per gram*
Heart muscle	2.88	4.85	13.79	11.90	49.98	5593
Casein	8.36	3.44	0.27	13.38	48.42	5240
Gelatin	10.05	3.40	0.11	15.30	42.73	4523

from the supplemented diets with those obtained from the basal ration alone the excretory products representing the protein supplements were computed.

The average results of the balances of matter and energy representing the different quantities of the proteins tested are presented in table 2.

The uniformity of the performance of the six experimental animals is indicated by the small probable errors of the mean results.

Of the nitrogen ingested as heart muscle at the low and high levels 3.9% and 4.5%, respectively, appeared in the feces, and 67.0% and 66.9% appeared in the urine.

Of the calories of heart muscle ingested at the low and high levels 2.1% and 5.4%, respectively, appeared in the

feces and 9.5% and 9.7% appeared in the urine. At the lower level 88.4% of the calories were metabolized, while at the higher level 84.9% of the calories ingested were metabolized.

Of the nitrogen ingested as casein at the lower and higher levels 3.5% and 3.2%, respectively, appeared in the feces, and 90.5% and 79.6% appeared in the urine.

TABLE 2

Metabolizability of meat protein, casein and gelatin

	HEART MUSCLE		CASEIN		GELATIN	
	1.5 gm.	3.0 gm.	1.5 gm.	3.0 gm.	1.5 gm.	3.0 gm.
Income						
Energy, calories	8.390	16.779	7.860	15.720	6.785	13.570
Carbon, grams	0.750	1.499	0.726	1.453	0.641	1.282
Nitrogen, grams	0.179	0.357	0.201	0.401	0.230	0.460
Outgo						
Feces						
Energy, calories	0.174	0.913	0.293	0.617	0.002	0.170
Carbon, grams	0.017	0.077	0.030	0.057	0.007	0.021
Nitrogen, grams	0.007	0.016	0.007	0.013	0.008	0.012
Urine						
Energy, calories	0.800	1.632	1.171	2.069	1.245	2.476
Carbon, grams	0.068	0.140	0.089	0.151	0.106	0.212
Nitrogen, grams	0.120	0.239	0.182	0.319	0.190	0.349
	±0.005	±0.004	±0.005	±0.005	±0.006	±0.006
Metabolizable						
Energy, calories	7.416	14.234	6.396	13.034	5.538	10.924
	±0.069	±0.066	±0.072	±0.105	±0.066	±0.085
Carbon, grams	0.665	1.282	0.607	1.245	0.528	1.049
	±0.004	±0.005	±0.005	±0.008	±0.006	±0.008
Energy, per cent	88.39	84.83	81.37	82.91	81.62	80.50
Carbon, per cent	88.67	85.52	83.61	85.68	82.37	81.83

Of the calories of casein ingested at the two stated levels 3.7% and 3.9%, respectively, appeared in the feces, and 14.9% and 13.2% appeared in the urine. The metabolizability of the energy of the casein at these levels was 81.4% and 82.9%, respectively.

Of the nitrogen ingested as gelatin at the lower and higher levels 3.5% and 2.6%, respectively, appeared in the feces, and 82.6% and 75.9% appeared in the urine.

Of the calories of gelatin ingested at the two stated levels 0.03% and 1.3%, respectively, appeared in the feces, and 18.3% and 18.2%, respectively appeared in the urine, thus leaving 81.6% and 80.5% as metabolized at the two different levels.

The foregoing results show that the metabolizability of the casein, as far as energy is concerned, was only slightly greater than that of gelatin, but that the metabolizability of the heart muscle was significantly higher than that of the casein or the gelatin. This was probably due in large part to the relatively higher fat content of the heart muscle (table 1). This difference in the fat content of the proteins tested is, however, without effect on the final computations of the respiration and energy factors, as will be explained below.

The derivation of the respiration and energy factors is set forth in detail in table 3. As in the previous paper (Kriss and Miller, '34) the computations are on the basis of pure protein.

Since the amounts of fat in the casein and in the gelatin were negligibly small, the values representing the carbon and energy contents of these proteins, in pure form, are as determined on the basis of water-free and ash-free substance. The values representing the nitrogen, hydrogen and oxygen content of pure casein, and the hydrogen and oxygen content of pure gelatin are from Matthews ('30, p. 117). The value for the nitrogen content of pure gelatin is as determined on the basis of ash-free, water-free and fat-free substance. The values representing the nitrogen, carbon, energy, hydrogen and oxygen content of heart muscle are as given by Loewy and Oppenheimer ('11) for meat protein ('Fleischeiweisz').

The values for nitrogen, carbon and energy of feces and urine per 100 gm. of pure protein were computed on the basis of the data given in table 2 in relation to the nitrogen content of the proteins. The corresponding values for hydrogen and oxygen of the feces and urine were computed from the figures of Loewy and Oppenheimer ('11) for the excretion products of meat in relation to the nitrogen content of the material.

TABLE 3

Determination of factors for computing the respiratory exchange and the heat production in the metabolism of meat protein, casein and gelatin

	HEART MUSCLE		CASEIN		GELATIN	
	1.5 gm.	3.0 gm.	1.5 gm.	3.0 gm.	1.5 gm.	3.0 gm.
Intake—computed per 100 gm. pure protein						
Energy, calories	563.09	563.09	594.10	594.10	522.59	522.59
Nitrogen, grams	16.65	16.65	15.70	15.70	17.70	17.70
Carbon, grams	52.38	52.38	54.90	54.90	49.37	49.37
Hydrogen, grams	7.27	7.27	7.00	7.00	6.80	6.80
Oxygen, grams	22.68	22.68	22.65	22.65	25.13	25.13
Outgo						
Feces						
Energy, calories	16.18	42.46	22.89	24.16	0.15	6.54
Nitrogen, grams	0.65	0.74	0.55	0.51	0.62	0.46
Carbon, grams	1.58	3.58	2.34	2.23	0.54	0.81
Hydrogen, grams	0.21	0.21	0.20	0.20	0.23	0.23
Oxygen, grams	0.89	0.89	0.83	0.83	0.94	0.94
Urine						
Energy, calories	106.67	108.64	97.48	98.52	111.92	122.31
Nitrogen, grams	16.00	15.91	15.15	15.19	17.08	17.24
Carbon, grams	9.07	9.32	7.41	7.19	9.53	10.47
Hydrogen, grams	2.66	2.66	2.51	2.51	2.83	2.83
Oxygen, grams	14.10	14.10	13.30	13.30	14.99	14.99
Metabolizable						
Energy, calories	440.24	411.99	473.73	471.42	410.52	393.74
Carbon, grams	41.73	39.48	45.15	45.48	39.30	38.09
Hydrogen, grams	4.40	4.40	4.29	4.29	3.74	3.74
Oxygen, grams	7.69	7.69	8.52	8.52	9.20	9.20
Intramolecular H_2O						
Hydrogen, grams	0.96	0.96	1.07	1.07	1.15	1.15
Oxygen, grams	7.69	7.69	8.52	8.52	9.20	9.20
Carbon oxidized to CO_2, grams	41.73	39.48	45.15	45.48	39.30	38.09
Hydrogen oxidized to H_2O, grams	3.44	3.44	3.22	3.22	2.59	2.59
Respiratory quotient	0.802	0.794	0.825	0.826	0.836	0.831
Liters O_2 required per gram urinary N	6.07	5.84	6.75	6.77	5.14	4.96
Liters CO_2 produced per gram urinary N	4.87	4.63	5.57	5.59	4.30	4.13
Calories metabolized per gram urinary N	27.52	25.90	31.27	31.03	24.04	22.84
Calories metabolized per liter O_2	4.532	4.433	4.632	4.581	4.673	4.601

All other calculations for the derivation of the respiration factors are self-explanatory.

The final results obtained with each of the proteins tested at the two different levels of feeding agree closely. The average results are as follows:

	Heart muscle	*Casein*	*Gelatin*
Respiratory quotient	0.798	0.826	0.834
Liters O_2 required per gram urinary N	5.96	6.76	5.05
Liters CO_2 produced per gram urinary N	4.75	5.58	4.22
Calories metabolized per gram urinary N	26.71	31.15	23.44
Calories metabolized per liter O_2	4.483	4.607	4.637

All values obtained for heart muscle agree very closely with the corresponding values of Loewy and Oppenheimer ('11) for meat protein. Their factors per gram of urinary nitrogen are 5.94 liters O_2, 4.75 liters CO_2 and 26.51 calories, giving the value of 4.463 calories per liter of O_2, and the R.Q. of 0.800.

The factors obtained for casein agree very closely with those obtained for this protein in the previous study by Kriss and Miller ('34). The latter values are 6.67 liters O_2, 5.47 liters CO_2, and 30.59 calories per gram of urinary nitrogen, with the R.Q. of 0.821 and the calorific value of respiratory O_2 of 4.586 per liter. This close agreement is especially significant, inasmuch as in the previous study the casein was fed exclusively, while in the present study the casein was fed as a supplement to a mixed diet.

The respiration and energy factors for gelatin per gram of urinary nitrogen are somewhat higher than those calculated by Rapport ('24) for this protein. The calorific value per liter of respiratory O_2 is slightly lower than that calculated by Rapport, while the R.Q.'s are almost identical.

When the factors per gram of urinary nitrogen of the three proteins are compared it is observed that they differ significantly from each other. The highest values are for casein and the lowest are for gelatin, with meat taking an intermediate place.

SUMMARY

Balances of nitrogen, carbon and energy were determined with six albino rats receiving first a mixed diet for maintenance, as the basal diet, and then the same diet supplemented, in different periods, with each of the three different proteins, namely, heart muscle, casein and gelatin in quantities of 1.5 gm. and 3.0 gm. each. The metabolizability of the proteins was determined from a comparison of the basal diet with each of the supplemented diets.

Of the total calories of the proteins ingested at the two levels the following average percentages were metabolized: heart muscle, 86.61%; casein, 82.14%; gelatin, 81.06%.

The following factors were determined for computing the respiratory exchange and the heat production in the metabolism of the different proteins, all values being expressed per gram of urinary nitrogen: for heart muscle, 5.96 liters of respiratory O_2, 4.75 liters of CO_2 and 26.71 calories; for casein, 6.76 liters of O_2, 5.58 liters of CO_2 and 31.15 calories; for gelatin, 5.05 liters of O_2, 4.22 liters of CO_2 and 23.44 calories. The factors for casein are in close agreement with those previously determined by Kriss and Miller. The factors for heart muscle agree very closely with those reported by Loewy and Oppenheimer for meat.

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BIOLOGICAL ASSAY OF LACTOFLAVIN WITH CHICKS

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FOUR FIGURES

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In a previous communication (Lepkovsky and Jukes, '36 a) a diet for chicks was described, deficient in lactoflavin, but fairly adequate in other respects. Norris, Wilgus, Ringrose, Heiman and Heuser ('36) have recently summarized their investigations on 'vitamin G' in poultry feeding. They used chick growth as a method for 'vitamin G' assay, and state that "the dried pork liver used as a reference was found to contain approximately 100 micrograms of flavin per gram. In view of this, the reference liver was given a value of 100 chick units of vitamin G per gram. Hence a chick unit of vitamin G is approximately equal to 1 microgram of flavin." In the present investigation, diets similar to those used by Norris and co-workers in 'vitamin G' assay were found to be deficient in both flavin and the 'filtrate factor' (Lepkovsky and Jukes, '36 b). Dried pork liver is a rich source of these factors, both of which are strongly growth promoting (Lepkovsky and Jukes, '35). In order to assay lactoflavin by chick growth it is desirable to use a diet which will respond only to crystalline lactoflavin. An attempt to produce such a diet for the quantitative assay of lactoflavin in foodstuffs is described below.

EXPERIMENTAL

a. Demonstration of a double deficiency in a diet based on unheated corn meal, wheat middlings and purified casein

Chicks were placed at hatching on a basal diet similar to that used by Wilgus, Norris and Heuser ('35). It consisted of ground yellow corn, 55 parts; flour wheat middlings, '20; acid-washed casein, 18;[1] bone ash, 2; cottonseed oil (Wesson oil), 4; cod liver oil, 1; manganous sulfate, tetrahydrated, 0.03; hexane extract of alfalfa meal, equivalent to 1% of alfalfa meal (diet 169).

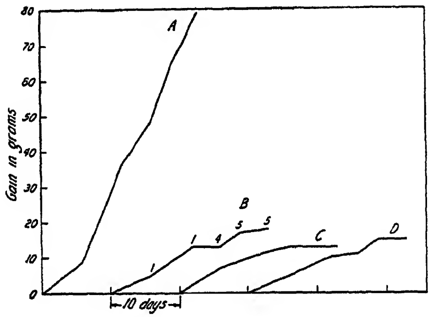

Fig. 1 Growth of chicks on diet 169 with various supplements. Six chicks were used in each group. The supplements fed to the chicks represented by curve A were lactoflavin and filtrate factor; curve B, lactoflavin; curve C, filtrate factor; curve D, basal diet (no supplement). The figures above curve B represent the number of chicks with dermatitis. No chicks in the other groups developed dermatitis.

The chicks were divided into four groups at the age of 14 days. The following supplements were used per 100 gm. of diet; group 1, 0.70 mg. of crystalline lactoflavin, prepared by Dr. S. Lepkovsky from whey adsorbate[2] as previously described (Lepkovsky, Popper and Evans, '35; Lepkovsky and Jukes, '36 a) plus 3 gm. of rice bran filtrate[2] (Lepkovsky, Jukes and Krause, '36); which had been treated three times with fuller's earth. The filtrate was found to be free from

[1] Supplied by the California Milk Products Co., Gustine, California.

[2] We are indebted to Vitab Products Inc., of San Francisco, for whey adsorbate and for the rice bran extract from which rice bran filtrate was prepared.

lactoflavin by testing it with rats. Group 2, 0.70 mg. of lactoflavin. Group 3, 3 gm. of rice bran filtrate. Group 4, no supplement. Group 1 grew rapidly and appeared normal in every way. Group 2 grew very slowly and developed typical 'chick dermatitis' (Lepkovsky, Jukes and Krause, '36). Groups 3 and 4 grew very slowly, but showed no symptoms of dermatitis. The results, illustrated in figure 1, made it evident that a diet for lactoflavin assay must be adequately supplemented with the filtrate factor.

b. Biological assay of lactoflavin with chicks

General methods in the care of chicks were previously described (Lepkovsky and Jukes, '35).[3] The birds were kept on ordinary mixed diet for 8 days following hatching, and were then placed on diet 96, low in lactoflavin, for from 6 to 10 days. At the end of this depletion period they were divided into experimental groups and placed on the test diets, which consisted of diet 96 plus the material to be assayed. Nine or ten chicks were used in each group. The starch and casein in the test diets were adjusted to maintain the nutritive ratio of the basal diet. An assay period of about 2 weeks was used.

Basal diet 96 had the following composition:

	parts
Yellow corn meal	30
Corn starch	23
Acid washed bran (Lepkovsky and Jukes, '36)	10
Washed casein[4]	22
Rice bran filtrate[5]	7
Sodium chloride	1
Ground limestone	1
Steamed bonemeal	1
Crude soybean oil[6]	3
Cod liver oil	2

Hexane extract of alfalfa meal, equivalent to 1% of alfalfa meal, evaporated on the diet.

[3] The F. E. Booth Co., San Francisco, kindly loaned battery brooders.

[4] See footnote 1, page 224.

[5] See footnote 2, page 224.

[6] Furnished by Allied Mills, Inc., of Peoria, Illinois.

Rice bran filtrate was prepared from rice bran extract as previously described (Lepkovsky, Jukes and Krause, '36), and was given five treatments with fuller's earth. The resulting preparation was found by assay to contain 20 units of the filtrate factor per gram (Jukes, '37). Seven per cent hence supplied an amount of the filtrate factor in excess of the amount required for maximal growth. The preparation was found to be free from lactoflavin by testing it with rats.

Acid-washed bran and soybean oil protected against gizzard erosions (Almquist and Stokstad, '37). The soybean

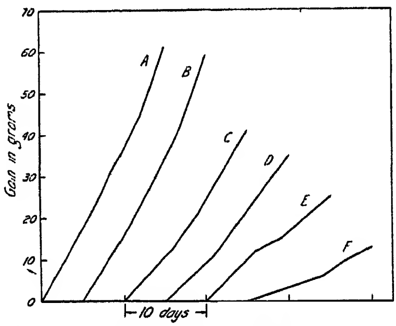

Fig. 2 Lactoflavin assay by chick growth, using diet 96, supplemented in the diet of the group represented by growth curve A with whey adsorbate 1, 3.0%; curve B, 2.0%; curve C, 1.0%; curve D, with crystalline lactoflavin, 0.28 mg. per 100 gm. of diet; curve E, 0.15 mg.; curve F, no supplement (basal diet). Nine chicks were used in each group.

oil was shown by chick tests with a 'purified diet' to furnish the anti-encephalomalacic factor of Goettsch and Pappenheimer ('36). Other details have been previously discussed (Lepkovsky and Jukes, '36 a).

The basal diet was tested by supplementing it with crystalline lactoflavin, with and without the addition of a vitamin $B(B_1)$ concentrate prepared from rice bran (Lepkovsky and Jukes, '36 a). The vitamin B concentrate did not cause additional growth. A whey adsorbate [7] (Jukes, '37) was fed to some of the groups in order to standardize it against lactoflavin. This adsorbate will be referred to as 'whey adsorbate 1.' Some results are illustrated in figure 2 in which the growth

[7] See footnote 2, page 224.

curves show that the response to graded suboptimal doses of lactoflavin was recti-linear, since in this experiment an addition of 0.00015% of lactoflavin gave 12 gm., and 0.00028% gave 22 gm. increase in growth over the basal diet. Two per cent or 3% of whey adsorbate 1 both gave almost identical maximal growth responses of 46 and 48 gm. over the basal diet. The linear response indicated that 0.0006% of the sample of lactoflavin used was the level which would just suffice for maximal growth. The lactoflavin was tested with rats, and 5 micrograms daily produced a slightly better growth response than is required for 1 Bourquin and Sherman unit.

The results of a number of experiments indicated that an assay period of about 2 weeks with chicks gives practically the same results as a longer period. The following results serve as an example:

DIET	GAIN IN 13 DAYS	GAIN IN 25 DAYS	RATIO OF GAIN IN 25 DAYS TO GAIN IN 13 DAYS
Basal + 0.7% whey adsorbate 1	31	78	2.5
Basal + 2.0% whey adsorbate 1	68	150	2.2
Basal + 2.5% whey adsorbate 1	72	160	2.2
Basal + 3.0% whey adsorbate 1	65	152	2.3
Basal	11	25	2.3

The effect of prolonged depletion of chicks was tried. Ten chicks were kept on the basal diet from 7 to 91 days of age. During this time the average gain in weight was 90 gm. At the end of this period there were five survivors. Three of the birds which died showed 'curled-toe' paralysis similar to that described by Norris, Heuser, Wilgus and Ringrose ('30–'31). The survivors were very emaciated and relucant to stand. The average weight was 139 gm. They showed practically no comb growth. Their heads had a peculiar crow-like appearance due to disproportionately large beaks. The beaks were, however, smaller than is usual for birds of this age. Two of the birds were placed on a supplement of 3% of whey adsorbate 1. They responded dramatically. Their activity greatly increased within 3 days. In 33 days they gained an

average of 194 gm. Their combs grew quite noticeably. Both often exhibited a rapid tremor of the head and neck. The birds remaining on the basal diet all died within 12 days. Similar results were obtained by injecting crystalline lactoflavin (fig. 3). Figure 4 shows the appearance of a normal bird in contrast.

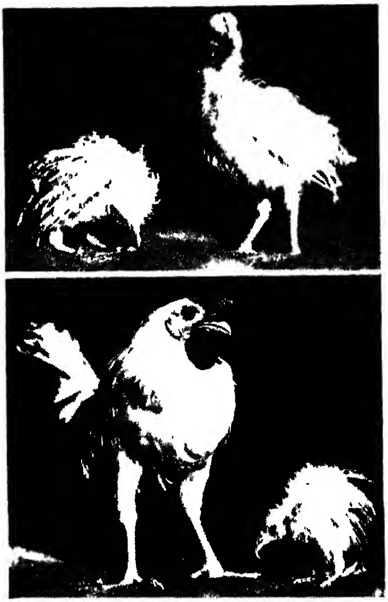

Fig. 3 The two chicks above were placed on basal diet 96 when 7 days old. At 97 days, the bird at the right received a single intramuscular injection of 1 mg. of crystalline lactoflavin dissolved in 2 cc. of 0.9% sodium chloride solution. Seventeen days later, when the picture was taken, its weight had increased from 101 to 190 gm. During the same period, the uninjected bird on the left had increased in weight from 89 to 96 gm.

Fig. 4 One hundred and fourteen-day-old chicks raised respectively on ordinary mixed diet and on diet 96.

Diet 96 was used for the assay of lactoflavin in feeding stuffs. A positive control diet consisting of diet 96 plus 2% of whey adsorbate 1 was employed, and the results were given an arithmetical treatment similar to that used in calculating the 'filtrate factor value' and 'filtrate factor unit' (Jukes and

Lepkovsky, '36; Jukes, '37). Table 1 summarizes various assays in terms of 'chick units of lactoflavin.' One chick unit is one-tenth of the amount which will just provide for maximal growth when fed daily to a chick during the assay period

TABLE 1

Distribution of lactoflavin in feeding stuffs. One chick unit is equivalent to about 1 modified Bourquin and Sherman rat unit

MATERIAL	PER CENT FED IN BASAL RATION	GROWTH RESPONSE	GROWTH ON POSITIVE CONTROL DIET	GROWTH ON BASAL DIET	LACTOFLAVIN CONTENT IN UNITS PER GRAM
Brewers' yeast 1	2	25	58	12	14
Young alfalfa shoots, dried at 40° (fall)	8 (dried basis)	47	55	15	10 (dried basis) 2.0 (fresh basis)
Lawn clippings dried at 40° (fall)	8 (dried basis)	41	55	15	8 (dried basis) 2.0 (fresh basis)
Dried skim milk	3	30	68	13	10
	5	33	48	20	10
	8	42	58	12	8
Alfalfa leaf meal	8	35	58	12	6
Dehydrated alfalfa meal	8	41	58	12	8
Wheat germ	10	28	55	15	3.1
	10	34	67	18	3.3
Soy bean meal	10	23	58	12	2.4
Sesame meal	10	20	58	12	1.7
Hempseed meal	10	20	55	15	1.2
Peanut meal	10	17	58	12	1.1
Cane molasses	5	10	58	12	....
Whey adsorbate 1 [1]	1	41	60	13	60
	1	37	53	9	64
	1	39	70	10	48
	1	57	75	18	68

[1] Sixteen and six-tenths milligrams fed daily to rats resulted in an average gain of 20 gm. in 4 weeks, i.e., the adsorbate supplied about 60 modified Bourquin and Sherman units (Dimick, Smith and Davis, unpublished) per gram. We are indebted to Mrs. M. I. Dimick of Vitab Products, Inc., for the rat assay.

under the conditions employed. The assay period lasted from the end of the second to the end of the fourth week of the chicks' age, hence the average age was 3 weeks. The average daily food consumption when diet 96 was adequately or nearly adequately supplemented with lactoflavin was about 10 gm.

Hence, as in the case of the 'filtrate factor unit' (Jukes, '37), the number of units of lactoflavin per gram of a supplement was calculated by dividing the net gain on the supplemented diet by the net gain on the positive control diet multiplied by the amount of the supplement in 1 gm. of the supplemented diet. One chick unit was approximately equal to 6 micrograms of the sample of crystalline lactoflavin used. The degree of purity of the sample is not known. One chick unit was approximately equal to a modified Bourquin and Sherman unit of 'vitamin G,' determined by rat growth on a diet deficient only in lactoflavin (Dimick, Smith and Davis, unpublished).

There is a marked difference in distribution between lactoflavin and the filtrate factor in common feeding stuffs. Filtrate factor assays (Jukes and Lepkovsky, '36; Jukes, '37) of some of the materials whose lactoflavin contents were also determined gave the following results.

MATERIAL	CONTENT IN UNITS PER GRAM OF	
	Filtrate factor	Lactoflavin
Brewers' yeast	13.0	14.0
Dried young alfalfa shoots	1.0	9.0
Dried lawn clippings	<0.2	8.0
Dried skim milk	3.0	9.0
Alfalfa leaf meal	1.3	6.0
Dehydrated alfalfa meal	3.0	8.0
Peanut meal	3.5	1.1
Cane molasses	6.0	Not detected
Whey adsorbate 1	<0.2	60.0

DISCUSSION

Wilgus, Norris and Heuser ('35) state that their basal diet "was seldom deficient in the antipellagric phase, and then so slightly that growth was not retarded." In the present investigation it was found that a similar basal diet was deficient in the 'chick antipellagric' (filtrate) factor to an extent which greatly retarded growth. The lack of agreement is probably due to differences is the filtrate factor content of the natural feeding stuffs used in the basal diets in the respective investigations. Birds on diet 169 did not show symptoms of dermatitis unless lactoflavin was added. Previous observations

(Elvehjem and Koehn, '35; Lepkovsky and Jukes, '35; Lepkovsky, Jukes and Krause, '36) have shown that dermatitis due to a deficiency of one member of the vitamin G complex is much more pronounced when the other members are supplied in ample amounts. Growth promotion, of course, is the function of all definitely characterized members of the vitamin B complex, and a mild deficiency of the 'filtrate factor' often results in retardation of growth without the appearance of dermatitis in chicks (Jukes, '37).

A short assay period minimizes the possibility of an outbreak of cannibalism which often occurs when chicks are confined to battery brooders for a long time. Depleted chicks and balanced groups must be used to obtain accurate results in a short-period assay. Balanced groups were selected at the end of the depletion period by dividing all the chicks into ten weight classes and taking one chick from each weight class to form a group.

The results in this investigation indicated that certain commonly used 'vitamin G' supplements, such as greens, alfalfa meal and dried milk are, in terms of similarly computed chick growth units, much richer sources of lactoflavin than of the filtrate factor. In ordinary poultry rations, this difference is compensated to some extent by the fact that the cereal grains and their by-products furnish appreciable amounts of the filtrate factor, although they are poor sources of lactoflavin.

Units of vitamins obtained by assay with rats are often based upon grams of gain per week. The chick unit used in this investigation and in previous work (Jukes and Lepkovsky, '36) is based upon percentage of maximal net growth obtainable, measured by the gain of a positive control group minus the gain of a group on the basal diet. The use of net growth rather than total growth compensates for 1) small amounts of the vitamin in the basal diet, inevitably present, since successful chick diets always include unpurified natural feeding stuffs, 2) vitamin reserves in the body of the chick. The use of a positive control diet minimizes effect of variations in growth rate in different hatches of chicks. There are great variations in growth rate at different times of the year in

chicks of the same strain. The following results indicate the extent of the variations which were encountered in the present investigation:

Experiment no.	1	2	3	4	5	6	7	8	9	10
Gain in 14 days on		68	65						55	
positive control diet	53	66	69	50	60	53	49	43	57	55

Gains of the other groups in each experiment were never greater than the gain of the positive control group or groups. In experiments in which the positive control group grew comparatively slowly, the growth of the assay groups was apparently proportionately slower.

The results of the experiment on prolonged depletion were in harmony with the observations of Bethke, Record and Wilder ('36) who found that pure flavin preparations prevented the occurrence of a leg disorder (Norris and co-workers, '30–'31; Bethke and co-workers, '30–'31).

SUMMARY

1. A diet similar to that used in 'vitamin G' assays (Norris and co-workers, '36) was found to be deficient in both lactoflavin and the filtrate factor. Addition of crystalline lactoflavin to the diet appeared to provoke dermatitis in chicks.

2. A basal diet for chicks is described which was used in the biological assay of lactoflavin by growth. Maximal growth under the conditions of assay required the addition of about 0.60 mg. of a crystalline preparation of lactoflavin to 100 gm. of the diet. The exact purity of the lactoflavin used was not known. The growth response to lower levels was roughly proportional to the amount of lactoflavin which was fed.

3. Symptoms of prolonged lactoflavin deficiency in chicks are described.

4. The lactoflavin content of some feeding stuffs is reported in terms of a chick unit which was based on chick growth, and was equivalent to one-tenth of the daily amount which will just provide for maximal growth under the conditions described. The unit was approximately equivalent to a modified Bourquin and Sherman unit of 'vitamin G.' Under the con-

ditions of the assay, the requirement of the chick for maximal growth was about 100 units per 100 gm. of diet.

5. Attention is drawn to the differences in distribution of lactoflavin and the filtrate factor is certain feeding stuffs.

The assistance of K. K. Miya and Maitland J. Wolfe is gratefully acknowledged.

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ASSAY PROCEDURE FOR VITAMIN K (ANTI-HEMORRHAGIC VITAMIN)

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That the absence of vitamin K in the diet of the chick leads to deficient blood clotting power, hemorrhages and anemia, has been shown in a number of papers (Dam, '35 a, '35 b; Almquist and Stokstad, '35 a, '35 b). Schonheyder ('36) has described the method of assay used by Dam and Schonheyder ('36). In brief, this method consists of rearing chicks on a normal diet for 2 weeks, then on a vitamin K deficient diet for at least 3 weeks at which time all animals are assumed to be depleted of vitamin K. Blood samples are taken from a carotid artery by cannula and centrifuged. The resulting plasma is diluted with Ringer's solution and the amount of a clotting agent necessary to cause coagulation of the plasma in approximately normal time is determined. Substances to be tested for vitamin K are then given by capsule to birds of the same group and these birds are tested for clotting time after several days. It appears to us that this method is unnecessarily complicated for practical purposes, lengthy from the standpoint of time required in obtaining depleted birds, and open to errors due to the introduction of a new factor, the clotting agent.

The preventive method used in our laboratory is based upon the occurrence of hemorrhage and upon simple measurement of blood clotting time. Striking differences in regard to hemorrhages and clotting time in various groups have frequently been obtained before the chicks were 2 weeks old. Some of

the precautions necessary in producing distinct deficiency at such an early date have already been mentioned (Almquist and Stokstad, '36).

The present paper deals with the assay procedure in detail and its application to the testing of various substances.

METHOD

Groups of newly hatched chicks were placed in metal, wire floored electrically heated battery brooders and given diet E as described in a previous paper (Almquist and Stokstad, '36). The substance to be tested was intimately mixed with the diet at suitable levels, in the case of solids by fine grinding and mixing, in the case of fat-soluble substances by solution in hexane and mixing the hexane solution thoroughly in the diet. The hexane soon evaporated leaving the substance uniformly dispersed in the diet.

Blood samples were taken from chicks by puncture of a main wing vein in such manner as to avoid cutting of muscular tissue and by allowing about 0.2 cc. of blood to fall into a clean glass vial. The vial was placed at once in a water thermostat at 38.5°C. and shaken by a constant speed mechanism. When the blood was no longer able to flow as the tube was shaken, it was considered to have clotted. The time of withdrawal of the blood sample and the time of clotting were recorded. The difference, in minutes, was taken at the clotting time. When this time was greater than 30 minutes, the blood sample was considered negative.

Since anemia had been observed as a portion of the general syndrome, it seemed advisable to include a study of hemoglobin levels as a possible aid in the procedure for determining adequacy of vitamin K supplements. Hemoglobin was measured by the Wu modification of the acid hematin method. The standard was a sample of chicken blood in which the hemoglobin level was determined by the oxygen capacity method. Comparison of the blood samples with the standard was made by a colorimeter.

RESULTS AND DISCUSSION

Results obtained during an assay of a vitamin K concentrate are given in table 1. The information in table 1 leads to several conclusions of interest.

1. Comparison of group 5 with group 6 shows that group 5 was somewhat deficient in vitamin K, giving evidence of prolonged clotting time and hemorrhage. Group 6 receiving 20% more vitamin K was not deficient. The procedure may therefore be regarded as capable of detecting slight deficiencies as well as complete deficiencies.

2. Prolonged clotting time was found in the first six groups at 1 week of age and was especially prevalent in the first four groups. This fact indicates rapid exhaustion of the vitamin K reserves of the newly hatched chick. It also indicates that the observations of clotting time may give significant results even earlier than observations of hemorrhage.

3. While prolonged clotting time was found at 1 week in deficient groups, hemorrhages were also noted in all of these groups at the same or a later date. The first two groups would probably have shown a high incidence of spontaneous hemorrhage had not most of the chicks died from bleeding as a result of the taking of blood samples. Thus the observation of hemorrhage leads ultimately to the same result as measurement of clotting time.

4. The average hemoglobin values are of special interest as they show that at an age of 1 week, when prolonged clotting time was found in a majority of the chicks in the first four groups but hemorrhage had affected only two of these chicks, the hemoglobin values were not appreciably different from those of the last three groups and particularly the last or non-deficient group. This evidence is in agreement with the statement of Schonheyder ('35) that deficient blood shows no abnormalities other than lack of clotting power. Our data show explicitly that avitaminosis K does not lead directly to anemia. The anemia reported in vitamin K deficient chicks (Dam and Schonheyder, '34; Holst and Halbrook, '33) must be regarded entirely as a consequence of hemorrhage.

TABLE 1

Relationship of blood clotting time, hemoglobin level and hemorrhage (eight chicks in each group)

GROUP NUMBER AND VITAMIN K CONCENTRATE, MILLIGRAMS PER KILOGRAM OF DIET	CLOTTING TIME TO NEAREST MINUTE, AT AGE OF			AVERAGE HEMOGLOBIN LEVEL, GRAMS PER 100 CC., AT AGE OF			HEMORRHAGIC SYMPTOMS, NUMBER OF CHICKS AT AGE OF			DIED FROM BLEEDING, NUMBER OF CHICKS AT AGE OF		
	1 week	2 weeks	3 weeks	1 week	2 weeks	3 weeks	1 week	2 weeks	3 weeks	1 week	2 weeks	3 weeks
0	15, 12 (6)[1]	(4)[1]	....	7.1	5.0	...	0	1	..	4	2	..
1	12, 17 (6)	6 (1)	....	7.6	...	...	0	3	..	4	2	..
2	9, 11 (6)	4, 5, 4, 7, 3 (2)	25, 3, 17, 3 (2)	7.2	5.9	6.8	1	3	0	1	1	2
3	(8)	23, 24, 27, 17 (2)	4, 2, 2, 8, 2	7.3	6.2	7.5	1	2	0	2	1	0
4	9, 7, 10, 8, 8 (3)	25, 3, 4, 6 (2)	11, 7, 4, 5, 3	7.2	5.8	7.0	0	1	0	1	1	0
5	3, 13, 10, 4, 4, 4, 10 (1)	28, 6, 2, 5, 8 (2)	3, 3, 16, 2, 3, 21	6.6	4.9	6.6	0	2	0	1	1	0
6	10, 2, 2, 1, 6, 2, 7, 3	2, 3, 1, 1, 3, 2, 1, 2	8, 4, 3, 2, 4, 3 3, 5	6.8	5.7	7.7	0	0	0	0	0	0

[1] Figures in parentheses refer to number of birds in which blood clotting time was greater than 30 minutes.

The lower hemoglobin levels in the second week are in agreement with the report of Harmon ('36) that chicks on normal diets show a minimum hemoglobin level at this age. To some extent, the hemoglobin values of the deficient groups may have been influenced by hemorrhage, however, they were not appreciably different from that of the last or non-deficient group. The hemoglobin values showed a rise at the third week, as would be expected (Harmon, '36). It is evident that determination of hemoglobin level does not constitute a necessary part of a vitamin K assay.

TABLE 2

Effect of soybean oil on blood clotting time of chicks approximately 2 weeks old

SUPPLEMENT	NUMBER OF CHICKS	HEMORRHAGIC SYMPTOMS NUMBER OF BIRDS	AVERAGE CLOTTING TIME IN MINUTES
None. Negative group control	10	4	12 (7)[1]
Hexane extract of alfalfa equivalent to 1%. Positive control group	10	0	1.7
Soybean oil 1%	7	0	15.6
Soybean oil, 2%	10	0	3.7
Soybean oil, 5%	8	0	1.3

[1] Figure in parentheses refers to number of birds in which blood clotting time was greater than 30 minutes.

5. The data suggest that at the third week the surviving chicks in groups three, four and five were spontaneously recovering since clotting time was restored toward normal. This may have been due to increased food intake, or a lowering of requirement for vitamin K as the birds became older. We have noted in many assays that the most clear-cut results are obtained with chicks up to 2 weeks of age. With older chicks, the syndrome develops more slowly and some may remain normal on deficient diets for comparatively long periods of time. Hence, we have adopted a 2-week test period, starting chicks on experimental diets as soon as hatched. This has a further advantage in conserving the basal diet and special supplement preparations.

To illustrate further the application of the preventive assay procedure, data on soybean oil as a vitamin K supplement are given in table 2.

In table 2 it is shown that 2% of soybean oil provided an adequate level of vitamin K, while 1% appeared slightly inadequate. This is the first report to our knowledge of the presence of vitamin K in soybean oil.

SUMMARY

1. A rapid procedure for assay of vitamin K supplements is described.

2. Determination of hemoglobin levels is unnecessary in such assay, since avitaminosis K is not a primary cause of anemia in chicks.

3. Vitamin K is present in soybean oil.

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SOURCES AND NATURE OF THE CHICK GIZZARD FACTOR

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Evidence for the existence of a fat-soluble factor required by the chick for the integrity of the gizzard lining has been presented in previous papers (Almquist and Stokstad, '36, '37). The existence of such a gizzard factor has also been the subject of reports by Kline, Bird, Elvehjem and Hart ('36) and Bird, Kline, Elvehjem, Hart and Halpin ('36). The latter workers have advanced the opinion that the factor is not fat-soluble, since attempts to extract it with ethyl alcohol and ethyl ether failed, and made the suggestion that the gizzard factor might prove to be identical with vitamin B_3.

It is the purpose of the present paper to provide further information on the distribution and properties of the chick gizzard factor.

METHODS AND RESULTS

The method followed was identical with that described in detail by Almquist and Stokstad ('37). The diet used was basal diet E supplemented with vitamin K in the form of alfalfa hexane extract treated with magnesium oxide to remove the gizzard factor. For simplicity, results have been condensed almost entirely to tabular form.

In table 1 are given the results of an experiment in which chicks were kept for 2 weeks on gizzard factor deficient diets and then for 4 weeks on supplemented diets.

The results of this experiment support the contention of Almquist and Stokstad ('36, '37) that the gizzard factor is fat-soluble. It remained in solution in hexane even after much fatty material had been removed by prolonged chilling at 0°C. followed by filtration at the same temperature. The solids removed were not active. The actual level of clarified wheat bran oil was 1.37% of the diet. This is the most active concentrate that has yet been obtained.

The result with dried normal gizzard lining is extremely interesting as it demonstrates the presence of the gizzard factor in the lining itself, and furnishes direct proof of the existence of an anti-gizzard-erosion factor. The result has been confirmed by other experiments.

TABLE 1

Average gizzard erosion score in chicks at 6 weeks of age

SUPPLEMENT	LEVEL	NUMBER OF CHICKS	AVERAGE GIZZARD EROSION, SCORE [1]	AVERAGE WEIGHT OF CHICKS
	%			gm.
None	......	12	0.88	342.1
Wheat bran hexane extract, clarified by chilling	30 (equiv.)	12	0.21	338.2
Solids removed from wheat bran extract by chilling	30 (equiv.)	13	0.92	327.0
Dried gizzard lining from normal gizzards	5	12	0.08	355.0

[1] This score is identical with the average number of plus signs per gizzard used in a former paper (Almquist and Stokstad, '37).

The growth results support the statement of Almquist and Stokstad ('37) that the gizzard factor is not a growth factor. There was no appreciable difference in the body weights attained by various groups although the gizzard erosions varied widely in severity between groups.

Further results on the distribution of the gizzard factor are given in table 2. In these experiments, chicks were placed on the various diets at 1 day of age and continued on these diets to an age of 4 weeks.

The results in table 2 offer further evidence that the gizzard factor is fat-soluble. The factor was found present in rice bran and in the hexane extract of rice bran but not in the hexane extracted rice bran. It was also found present in soybean oil which was a richer source than the soybean meal. Commercial soybean meals carry several per cent of the oil.

TABLE 2

Average gizzard erosion scores in chicks at 4 weeks of age

SUPPLEMENT	LEVEL	NUMBER CHICKS	AVERAGE GIZZARD EROSION, SCORE	AVERAGE WEIGHT OF CHICKS
	%			gm.
None	......	8	1.12	220.0
Rice bran [1]	15	8	0.62	237.6
Rice bran [1]	25	11	0.45	214.5
Rice bran, hexane extracted [1]	15	8	0.94	218.6
Hexane extract of rice bran	30 (equiv.)	8	0.25	215.0
Kale, dried at 0°C.[1]	15	11	0.45	225.2
Kale, dried at 0°C.[1]	20	10	0.10	214.0
Barley, ground [1]	25	9	0.72	231.1
Alfalfa, dried [1]	20	11	0.32	146.4
Soybean meal [1]	15	10	0.40	225.0
None	......	10	1.15	213.5
Peanut meal [1]	25	12	0.46	238.3
Sesame meal [1]	25	7	1.00	258.6
Hempseed meal [1]	25	10	0.05	247.5
Linseed meal [1]	25	6	1.17	81.7
Soybean meal [1]	25	11	0.23	....
Soybean oil no. 1 [1]	5	12	0.21	219.2
Soybean oil no. 1 [1]	10	8	0.20	211.0
Soybean oil no. 2 [1]	5	11	0.54	237.2
Soybean oil no. 2 [1]	10	8	0.19	216.3
Soybean oil no. 2, heated with ethyl alcohol [1]	10	10	0.85	204.2
Soybean oil no. 2, heated in vacuum for 24 hours at 120°C.[1]	10	10	0.90	223.0
Soybean oil no. 1, alcohol soluble fraction	10	8	1.38	217.0
Soybean oil no. 1, alcohol insoluble fraction [1]	10	8	0.86	225.0
Wheat bran [1]	25	10	0.45	256.0
Wheat bran, autoclaved at 120°C. for 24 hours [1]	25	10	1.10	230.0
Wheat bran, heated in vacuum at 120°C. for 24 hours [1]	25	9	1.33	221.0

[1] Used in replacement of an equal quantity of polished rice.

Hempseed meal had a strikingly unique effect on gizzard linings in addition to protecting them from erosions. These linings differed from all others that have been observed in that they were almost completely smooth and free from the ridges or folds usually found in a normal gizzard lining. Almquist and Stokstad ('37) have shown the presence of the gizzard factor in the hexane extract of hempseed. This extract, however, does not produce the peculiar smooth lining appearance noted with hempseed meal.

Evidence for destruction of the gizzard factor by alcohol is seen in the experiments with soybean oil heated to a refluxing temperature with ethyl alcohol, and with soybean oil that had been extracted several times with cold, 95% ethyl alcohol. Negative results were also obtained with the alcohol extract of soybean oil. These findings are in agreement with our previous experience in attempts to extract the gizzard factor. The use of ethyl and methyl alcohols, ethyl ether, or acetone has uniformly resulted in non-potent preparations indicating some destructive influence of these solvents.

The results with heated or autoclaved wheat bran and with heated soybean oil are in agreement with the report of Almquist and Stokstad ('36, '37) and Kline et al. ('36) that the factor is adversely affected by heating.

The average body weights of chicks given in tables 1 and 2 indicate definitely, in agreement with Almquist and Stokstad ('37), that the gizzard factor is not a growth factor. On the other hand, it has been stated by Kline et al. ('36) that absence of the gizzard factor leads to very poor growth, probably due to poor absorption. Further implications in regard to the effect of the gizzard factor on growth are to be found in the paper by Bird et al. ('36). Such a statement is difficult to understand in view of the fact that the gizzard is not an organ of absorption. Fritz, Burrows and Titus ('36) have shown with gizzardectomized fowls that the gizzard is not essential when fine ground feed is eaten.

Since the factor has been clearly demonstrated to be fat-soluble, in agreement with the first report from this laboratory, the supposition that the gizzard factor may be 'vitamin B_3' or any member of the vitamin B complex cannot be reconciled with the known properties of the factor. It seems possible, however, that more than one dietary factor may be concerned in the prevention of gizzard erosion.

SUMMARY

1. Additional information is presented on the distribution of the chick gizzard factor.
2. The gizzard factor is unstable to heat and to ethyl alcohol.
3. The gizzard factor is present in the gizzard lining.
4. The gizard factor has no noticeable relation to the rate of growth of chicks.

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VITAMIN A ACTIVITY OF BUTTERS DETERMINED BY VARIOUS METHODS [1]

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ONE FIGURE

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The vitamin A values of certain Kansas State College butters, determined by several methods developed in the past decade for quantitative estimation, are herein reported. Findings of 1) vitamin A and 2) carotene determinations were combined to estimate total vitamin A values and compared with 3) vitamin A values obtained by the single feeding method.

The work of Rosenheim and Drummond ('25) with $AsCl_3$ and Carr and Price ('26) with $SbCl_3$ led to numerous investigations of the resulting blue color as a test for vitamin A, often measured with a tintometer and also recently by means of the visual spectrophotometer (Morton, '35). Suitable preparation of the non-saponifiable portion (Baumann and Steenbock, '31, '33 b; Booth et al., '33) makes the Carr-Price test useful for butter.

Morton and Heilbron ('28) observed the presence of a specific absorption band at 328 mμ in oils, the intensity of the absorption being proportional to the vitamin A concentration.

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This method is suitable (Morton and Heilbron, '30; Baumann and Steenbock, '33 a) for study of the absorption maxima in butter.

Moore ('30) showed that the plant pigment carotene was converted into vitamin A in vivo. The determination of both vitamin A and its precursors is necessary for total vitamin A value in foods like butter which contain both. Carotene has been determined with the visual spectrophotometer (Baumann and Steenbock, '33 a) in melted butter fat or in butter dissolved in petroleum ether and also in the non-saponifiable portion dissolved in petroleum ether (Gillam and Heilbron, '35; Shrewsbury and Kraybill, '33), methods which permit the use of extinction coefficients (Skellysolve as solvent) based upon the international standard (Peterson et al., '37).

Biological assays, although time consuming, are considered valuable, to confirm results obtained in other ways, and fundamental, to give quantitative figures for the nutritive value of the vitamin and its precursor or precursors as used by the animal. The recently developed single feeding method (Sherman and Todhunter, '34), a modification of the widely used procedure of Sherman and Munsell ('25) has been found suitable for measuring the total vitamin A value of a product.

METHODS

The experimental butters were prepared in the Department of Dairy Husbandry, churned from sweet cream of 24-hour collections made on the first, fifth and thirtieth days of lactation and stored at 0°C. until used for experiment. The 'commercial sample' of butter was churned from milk delivered from neighboring farms and sold by the college dairy in the early part of February. Only this commercial sample contained added coloring matter, a vegetable compound which did not interfere with tests used, as shown by suitable checks.

Determination of vitamin A. 1. $SbCl_3$ spectrophotometric method. The unsaponifiable portion of each butter was prepared essentially as described by Morgan and associates ('35), using duplicate samples of 5 gm. of colostral butters or 15 gm.

of other butters. In preparing the solution for the spectrophotometer the proportion used by Gillam ('35) was maintained: 0.5 ml. of the unknown solution and 4.5 ml. of anhydrous $SbCl_3$ solution, prepared according to Wokes and Willimott ('27), were mixed and read against pure chloroform in less than 30 seconds at 583 mμ and 620 mμ, $E\left\{{}^{1\%}_{1\,\text{cm.}}\right.{}^{\text{'pure' vitamin A}}$ being equal to 2600 and 5000 (Morton, '35), respectively. Calculations were made by use of the Beer-Lambert law. Strain ('35) has stated that carotene gave a color reaction with $SbCl_3$, but with different maxima. This, however, was for concentrated solutions. That absorption here measured was due to vitamin A rather than to carotene was shown by running a control using an equivalent amount of carotene dissolved in Wesson oil in the above procedure in which case no color developed.

2. Spectrographic method. Duplicate samples were prepared (Baumann and Steenbock, '33 a; Semb, Baumann and Steenbock, '34). The vitamin A preparations, dissolved in methyl alcohol, sealed in an atmosphere of nitrogen, were kept in the dark until absorption spectra could be produced. The solutions were photographed with a Bausch and Lomb quartz spectrograph equipped with a Hilger rotating sector disc and a quartz biprism. The light source was a Ni-Fe arc, and the light was diffused through a frosted quartz plate. Values for $\log \frac{I_o}{I}$ at 328 mμ were obtained from duplicate plates and used in computing the value of $E\left\{{}^{1\%}_{1\,\text{cm.}}\right.{}^{\text{butter}}$, which were then compared with the accepted extinction coefficient for vitamin A, $E\left\{{}^{1\%}_{1\,\text{cm.}}\right.{}^{\text{'pure' vitamin A}}$, 328 mμ = 1600 (Carr and Jewell, '33) to calculate percentage of vitamin A in the butters (table 1). Corrections for the readings at 328 mμ have been suggested. De ('35) made spectrograms before and after exposing the sample to ultra-violet light for 3 hours and took the difference between the two absorptions to calculate vitamin A content. Morton ('35) described methods of correction and pointed out the interference of carotene and xanthophyll when chloroform is the solvent. However, as readings obtained when methyl alcohol is used as a solvent are usually accepted without correction, they are so presented here.

TABLE 1

Vitamin A and carotene determinations on butter, obtained by chemical and physical methods

BUTTER SAMPLES		VITAMIN A					β-CAROTENE			
		Spectrophotometric method, $SbCl_3$			Spectrographic method		Spectrophotometric method			
		620 mμ	583 mμ		328 mμ		480 mμ	470 mμ	455 mμ	
Source	Butter fat	$E^{1\%}_{1\,cm.}$ 'pure' vit. A = 5000	$E^{1\%}_{1\,cm.}$ 'pure' vit. A = 2600	Vitamin A	$E^{1\%}_{1\,cm.}$ 'pure' vit. A = 1600		$E^{1\%}_{1\,cm.}$ β-carotene = 2120	$E^{1\%}_{1\,cm.}$ β-carotene = 2000	$E^{1\%}_{1\,cm.}$ β-carotene = 2270	Carotene
	%	$E^{1\%\ butter}_{1\,cm.}$	$E^{1\%\ butter}_{1\,cm.}$	%	$E^{1\%\ butter}_{1\,cm.}$	*vitamin A %*	$E^{1\%\ butter}_{1\,cm.}$	$E^{1\%\ butter}_{1\,cm.}$	$E^{1\%\ butter}_{1\,cm.}$	%
Commercial	80.0	0.01560	0.01183	0.000383	0.01234	0.000771	0.00329	0.00334	0.00384	0.000164
Holstein 197										
Colostrum	89.5	0.07325	0.04591	0.001613	0.12010	0.007500	0.03788	0.03788	0.04222	0.001847
5th day	83.5	0.02200	0.01653	0.000506	0.01146	0.000710	0.00432	0.00444	0.00520	0.000218
30th day	84.0	0.01280	0.00775	0.000277	0.00919	0.000568	0.00583	0.00570	0.00663	0.000284
Jersey 342										
Colostrum	79.5	0.04115	0.02501	0.000892	0.08010	0.005000	0.05975	0.05950	0.06900	0.002945
5th day	90.0	0.02380	0.01510	0.000528	0.01975	0.001228	0.02251	0.02238	0.02624	0.001112
30th day					0.00644	0.000396	0.00225	0.00238	0.00329	0.000123
Jersey 378										
Colostrum	84.5	0.05900	0.04030	0.001365	0.06885	0.004297	0.04566	0.04534	0.05384	0.002264
5th day	91.0	0.01805	0.01258	0.000423	0.00978	0.000605	0.01024	0.01062	0.01226	0.000518
30th day	86.0	0.00785	0.00525	0.000179	0.00719	0.000443	0.00738	0.00750	0.00824	0.000362

Determination of carotene. Portions of 5 or 10 gm. of butter were dissolved directly in 25 ml. of petroleum-ether (Shrewsbury and Kraybill, '33). No correction was necessary for the butter fat dissolved in the Skellysolve, as determined by readings on samples decolorized with norite. The solution was filtered, made up to 100 ml., and read in a visual spectrophotometer at 455, 470 and 480 mμ. The extinction coefficients, $E\{^{1\%}_{1\,cm.}{}^{\beta\text{-carotene}} = 2270$, 2000 and 2120, respectively, determined by Peterson et al. ('37) for β-carotene in Skellysolve, were used. Gillam and Heilbron ('35) suggested that the xanthophyll be separated from the carotene by partition between 90% methyl alcohol and light petroleum ether. Gillam ('34) found the proportion of xanthophyll to carotene to be fairly constant in butter fat and to represent a ratio of 1:14 by weight, or approximately 6% of the total yellow coloring matter. In later routine work he did not perform the separation but simply corrected for the xanthophyll. Other authors feel that it is unnecessary to correct for xanthophyll, and carotene figures in this study are presented uncorrected.

Determination of total vitamin A value. The single feeding method of Sherman and Todhunter ('34) was employed for biological assay. Young rats of Wistar Institute strain, weighing 38 to 40 gm., were depleted of vitamin A reserves (average depletion period = 41 days) and divided into experimental groups of ten or more animals. Carotene (international standard for vitamin A held for the Health Section of the League of Nations by the National Institute for Medical Research, Hampstead, London, N.W. 3) secured through the Bureau of Chemistry and Soils, United States Department of Agriculture, was dissolved in cottonseed oil (Wesson oil, Baumann and Steenbock, '33 b) and stored in the dark below 0°C. until needed. Portions of this solution equivalent to 34 γ, 17 γ and 10 γ pure β-carotene were given as single feedings. Composite growth curves (fig. 1) were plotted so that areas could be measured in the usual way with the planimeter and comparisons made with the carotene reference curves.

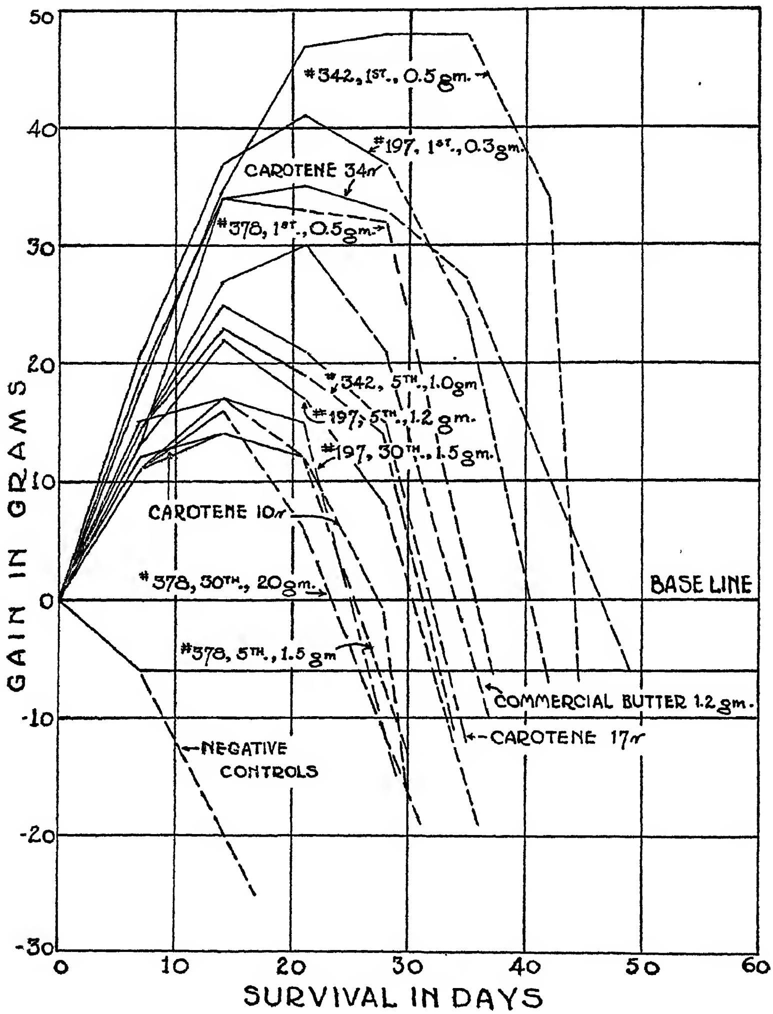

Fig. 1 Average weight curves of rats, previously depleted of body store of vitamin A, to which single feedings of butter and carotene have been given, as indicated.

DISCUSSION

Results with $SbCl_3$ gave information (table 1) regarding the relative richness in vitamin A of the various samples and definitely distinguished the butters of high from those of low vitamin A content. In agreement with the work of Morgan et al. ('35), who have discussed the limitations of the $SbCl_3$ method, results obtained from the 583 mμ readings were in every case higher than those from 620 mμ. According to Morgan et al. ('35) vitamin A values obtained with $SbCl_3$ tend to represent lower limits on account of inhibition while values obtained at 328 mμ indicate upper limits. Our findings are in accord with this for every butter tested, the values at 328 mμ being invariably distinctly higher than those obtained with $SbCl_3$.

The butters produced on the first day of lactation were richest in β-carotene. The commercial butter contained more β-carotene than that produced by cow 342 on the thirtieth day of lactation but less than the thirtieth day butters of the other two cows.

Table 2 summarizes 1) data obtained by biological assay, used to estimate the total vitamin A value of the butters, and 2) data from feeding the international standard β-carotene used for the carotene determinations. The areas under the composite curves have been shown by the originators of the method to be directly proportional to the vitamin value of the single feeding. The results of feeding three levels of carotene indicated on the table, show that this expected ratio of areas holds for the animals of the present series, confirming the validity of the method as used in our laboratory.

A summary of the findings obtained from the preceding tables expressed in gamma and as international units (Nelson, '35) is presented in table 3. Total vitamin A value is recorded in three ways: 1) the summation of the results from the $SbCl_3$ tests and from the carotene determinations, 2) the summation of results obtained from the intensity of absorption at 328 mμ and from the carotene determinations and 3) the biological assay findings. The three sets of figures show interesting

TABLE 2

Total vitamin A value determined by biological assay

SAMPLE	RATS USED		AMOUNT FED	AVERAGE LENGTH OF SURVIVAL	AREA	RESULTS PER GRAM OF BUTTER FED		VITAMIN A VALUE OF BUTTER
	Number	Average weight when depleted				Area	Value in β-carotene	
		gm.	*gm.*	*days*	*sq.in.*	*sq.in.*	γ	%
Commercial	9	94	1.2	37	8.25	6.88	16.99	0.001812
Holstein 197								
Colostrum	13	92	0.3	41	13.40	44.67	110.33	0.011769
5th day	8	95	1.2	36	5.75	4.79	11.83	0.001262
30th day	10	93	1.5	29	4.19	2.80	6.92	0.000738
Jersey 342								
Colostrum	11	92	0.5	44	17.77	35.54	87.78	0.009363
5th day	11	91	1.0	34	6.41	6.41	15.83	0.001689
Jersey 378								
Colostrum	13	93	0.5	37	10.00	20.00	49.40	0.005369
5th day	10	94	1.5	30	4.36	2.91	7.19	0.000767
30th day	10	94	2.0	31	3.45	1.73	4.27	0.000455
Carotene	11	91	10 γ	30	4.14	1 sq.in. = 2.41 γ pure β-carotene		
Carotene	23	92	17 γ	35	6.73	1 sq.in. = 2.48 γ pure β-carotene		
Carotene	9	92	34 γ	47	13.69	1 sq.in. = 2.53 γ pure β-carotene		
Negatives	17	93		17		Av. = 2.47 γ		

TABLE 3

Summary of total vitamin A values per gram of butter fat and per 24-hour yield obtained by chemical, physical and biological methods

SAMPLE	VALUES OBTAINED BY								
	$SbCl_3$ + carotene determinations			328 mμ + carotene determinations			Biological assay		
	Per gram butter fat		Per 24-hour yield	Per gram butter fat		Per 24-hour yield	Per gram butter fat		Per 24-hour yield
	γ	I.U.	I.U. (1000 S)	γ	I.U.	I.U. (1000 S)	γ	I.U.	I.U. (1000 S)
Commercial	6.98	11		11.83	18		22.65	35	
Holstein 197									
Colostrum	41.86	65	28	105.81	165	72	131.50	205	89
5th day	8.85	14	15	11.29	18	20	15.11	24	26
30th day	6.91	11	14	10.37	16	20	8.79	14	18
Jersey 342									
Colostrum	50.73	79	31	102.40	160	63	117.77	184	73
5th day	19.04	30	15	26.82	42	21	18.77	29	14
Jersey 378									
Colostrum	43.55	68	8	78.25	122	15	63.54	99	12
5th day	10.73	17	12	12.73	20	14	8.43	13	9
30th day	6.57	10	9	9.64	15	13	5.29	8	7

correspondence for results obtained in diverse ways. The second summation most often includes the highest figures for total vitamin A value whereas the first summation often includes the lowest of the three figures for any one sample. By any of the three methods, the colostral butters are shown to contain butter fat of highest vitamin A value, which is consistent with the findings of other workers (Dann, '33; Semb et al., '34; Gillam et al., '36). In the colostral butters studied, 20 to 40% of the total vitamin A value appeared to be due to the carotene and in some later butters a good proportion of the vitamin A value was supplied by precursors. The commercial butter had one of the lower carotene contents, accounting for about 19% of the total vitamin A value. This butter was churned from milk delivered at the dairy from neighboring farms on which the rations fed may have been deficient in good quality roughage as a result of the drought, whereas the other samples were made from milk of cows on well-balanced rations including liberal amounts of alfalfa hay and silage. Baumann and Steenbock ('33 a) found that the carotene in a series of Wisconsin butters, prepared throughout the year, accounted for an average of 15% of the vitamin A value, and later Baumann et al. ('34) reported large proportions of the vitamin A value to be due to carotene, about half for some Jersey samples and less for Holstein samples. Gillam et al. ('36) investigated the carotene and vitamin A contents of the colostrum of six cows kept under typical English conditions and reported well over half the vitamin A value due to carotene in five instances.

Data from dairy records were used to compile yields (table 3). Cow 378 was slow in starting her lactation and her yield was considerably lower than the other cows which accounts for the apparently low total production of vitamin A on the first day of lactation. Other cows furnishing samples for this experiment produced between 60,000 and 90,000 I.U. of vitamin A value on the first day of lactation. As lactation progressed the total daily yield of butter fat increased but the vitamin A value of the butter fat dropped so that the total

output of vitamin A value on the thirtieth day of lactation was distinctly lower. Dann ('33) found extreme differences between the vitamin A values of colostrum and standard milk, determined by colorimetric methods, and concluded that a calf receives on the first day of life supplies of vitamin A greater than later milk could give in 20 to 50 days. The present investigation of butter shows that the total vitamin A value of the colostrum of the first day of lactation may be four or five times as great as that of milk of the thirtieth day for the same cow.

SUMMARY AND CONCLUSIONS

Vitamin A values were determined for a 'commercial' winter butter and for butters made from composite 24-hour samples of the secretions of the first, fifth and thirtieth days of lactation of three individual cows, a Holstein and two Jerseys. The vitamin A content was estimated by measuring intensity of the blue color, obtained with the $SbCl_3$ reagent added to the unsaponifiable fraction, in a visual spectrophotometer and also the absorption spectra at 328 mμ with a quartz spectrograph (courtesy physics department, Iowa State College). The amount of carotene was measured spectrophotometrically with petroleum ether (Skellysolve) as the solvent. The vitamin A value, or combined activity of vitamin A and precursors, was determined by the single feeding method of biological assay, giving results expressed as international units of vitamin A, by reference to curves of animals fed standard β-carotene.

The $SbCl_3$ tests yielded information regarding the relative amounts of vitamin A in the various samples, distinguishing the butters of high from those of low vitamin A content. These findings with $SbCl_3$ on butter tended to represent definitely lower limits of vitamin A content whereas the results at 328 mμ represented the upper limits. The more satisfactory measurements at 328 mμ showed that the butter fats produced on the fifth day of lactation contained from one-fourth to one-tenth the amount of vitamin A per gram found in that produced by the same cow on the first day. Butter fats

produced on the thirtieth day of lactation showed further decreases in vitamin A content. The sample of commercial butter had a vitamin A content similar to those of later butters.

Readings with the visual spectrophotometer showed that the carotene contents of the colostral butters were definitely superior to those of the other butters. The carotene contents dropped during the course of lactation, as indicated by the tables. The carotene content of the commercial butter was similar to those of thirtieth day samples studied.

Biological assay showed the butter fat of colostrum to be richest in total vitamin A value for each cow studied. Butter fats produced on the fifth day of lactation were only one-sixth to one-ninth as potent while those produced on the thirtieth day of lactation were even lower in vitamin A value, as indicated.

Total vitamin A values per gram of butter fat, determined by the single feeding method, were compared to the summations of values obtained by chemical and physical methods, namely, $SbCl_3$ plus carotene and 328 mμ plus carotene. In some cases the summations including vitamin A determined with $SbCl_3$ were similar to the biological results while in other instances these summations were distinctly lower which is consistent with the view that the $SbCl_3$ readings represent lower limits for vitamin A content. The summations including the determinations of vitamin A at 328 mμ plus carotene values were on the whole in better agreement with the results of biological determinations of total vitamin A activity. Results of determinations at 328 mμ plus β-carotene values compare well with results of biological determinations by the single feeding method, and apparently account for most of the vitamin A value of the butter fats examined.

Results from these methods show that two samples of butter fat from colostrum contained about 175 international units of vitamin A value per gram and the other more than 100 units. Samples produced by the same cows on the fifth day of lactation were found to contain only one-fifth to one-ninth this amount. By any of the three methods of measurement, butter

fat of the thirtieth day of lactation was lower in vitamin A value than that of the fifth day of lactation. Total daily yields of vitamin A value were calculated for the individual cows; two of these produced between 60,000 and 90,000 international units on the first day of lactation while the third had a lower production. As lactation progressed the vitamin A value of the daily yield of butter fat dropped, that of the fifth day being lower than that of the first and the output on the thirtieth day showing some further decrease.

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THE INFLUENCE OF SOME COMMONLY USED SALT MIXTURES UPON GROWTH AND BONE DEVELOPMENT OF THE ALBINO RAT [1]

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A suitable mixture of inorganic salts forms a necessary part of any experimental diet composed of purified foodstuffs if this diet is to be adequate for normal nutrition. Early reports of attempts to prepare rations consisting wholly of purified foodstuffs indicated this need. The various combinations of salts employed by such workers as Lunin (1881), Socin (1891), and Hall (1896), were in large measure suggested by the analyses of milk by Bunge (1874). These investigators, as well as others, tried to construct mixtures that would provide the essential inorganic constituents of milk, and a wide variety of chemical compounds appears to have been used. A typical mixture is that recorded by Socin:

	gm.
Potassium carbonate	12.5
Calcium phosphate	20.3
Calcium carbonate	7.1
Magnesium chloride	2.7

Another early one, that of Munk (1893) shown below, was modelled after the 'Fleischasche' analyses of Wolff (1871,

[1] A preliminary report was presented before the American Institute of Nutrition at the Detroit meeting, April, 1935.

The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington.

1880), and is, as a result, quite different from those based on Bunge's milk analyses.

	gm.
Potassium phosphate	70
Sodium chloride	15
Calcium phosphate	6
Magnesium phosphate	8
Iron oxide	0.5

The investigators who used these mixtures were interested in a variety of metabolism experiments which need not be reviewed here. It is quite evident from the reports, however, that the partial failure of many studies was attributed to inadequacies in the salt mixture used, and some of the later workers attempted to correct the deficiencies by adding the ash of some animal tissue to the various combinations of inorganic salts. For example, Falta and Noeggerath ('06) added milk salts and the salts of horse serum to the mixture described by Socin.

In one of the early publications from this laboratory (Osborne and Mendel, '11), three salt mixtures are mentioned. The first was essentially that introduced by Röhmann in 1902 with the addition of ferric citrate, and differed from some of the others in that it included several organic salts. The second was suggested by the work of McCollum ('09) who supplemented the purified protein, fat, and carbohydrate of his experimental diet with 5% ash of milk, 2% calcium phosphate, and 1% sodium chloride with the addition of ferric chloride 'at intervals.' The third was a modification of that described by Henriques and Hansen ('05) with ferric citrate as an additional component.

The composition of these mixtures as used by Osborne and Mendel is shown in table 1.

In 1911, Osborne and Mendel began their studies of the relative nutritive value of proteins as determined by growth. As they reported at that time, earlier trials with purified protein, fat, carbohydrate and modified Röhmann salts had been successful only in maintaining the weight of experimental animals. On the other hand, growth had resulted when they fed a paste of milk powder, lard and starch. They accordingly turned their attention to the non-protein constituents of

milk. The product designated as 'protein-free milk' was prepared and, although it contained lactose in addition to the milk salts, was administered with the intention of supplying the needs of the animals for inorganic salts.

The success that followed the substitution of 'protein-free milk' for the salt mixture that had previously been used was so marked that Osborne and Mendel decided to investigate this particular fraction further in an attempt to explain the improvement. As they pointed out in 1912, it seemed possible that the difference might be due to the fact that the mixture of pure salts did not "represent precisely the composition of the milk salts as they occur in their natural medium," and

TABLE 1

Salt mixtures used by Osborne and Mendel in early investigations

SALT MIXTURE I		SALT MIXTURE II		SALT MIXTURE III	
	gm.		*gm.*		*gm.*
$Ca_3(PO_4)_2$	10.0	Ash of milk	60.6	NaCl	33.4
K_2HPO_4	37.0	$Ca_3(PO_4)_2$	24.2	KCl	33.4
NaCl	20.0	NaCl	12.1	Bone ash	25.1
Na citrate	15.0	Fe citrate [1]	3.1	Na_2CO_3	6.7
Mg citrate	8.0		100.0	Fe citrate [1]	1.4
Ca lactate	8.0				100.0
Fe citrate [1]	2.0				
	100.0				

[1] Fe citrate added by Osborne and Mendel.

that "a mixture of crystalline salts containing the various ions present in the protein-free milk might present an opportunity for an unequal rate of absorption of the inorganic salts from the ingested food." Consequently, they prepared 'artificial protein-free milk' (Osborne and Mendel, '12) by dissolving inorganic salts in a water solution of hydrochloric, phosphoric, citric, and sulfuric acids; lactose was added and the mixture was evaporated and dried. When this product was used to replace 'protein-free milk' in the food, growth was superior to that obtained with salt mixture I in the diet, but was not as satisfactory as that observed when natural 'protein-free milk' was used. The explanation of the superiority of the diets that contained 'protein-free milk' over those

containing the artificial product has been discussed elsewhere (Osborne and Mendel, '16, '17 a, '17 b). The significant fact in connection with the present paper is that 'artificial protein-free milk,' prepared without the addition of lactose, has been known for many years as Osborne and Mendel salts ('17 c, '19).

This salt mixture, as well as many others that have been described, has been widely used to supply the inorganic constituents in rations composed of purified foodstuffs. In some laboratories it has been found inconvenient to prepare the Osborne and Mendel salt mixture as they described it and, as a result, two modifications (Hawk and Oser, '31; Wesson, '32) have been suggested, both prepared by mixing appropriate dry salts. In each case the relative proportions of the elements are substantially the same as in the original.

The essential differences in elementary composition between these and some of the other well-known salt mixtures (McCollum and Davis, '14; Steenbock and Nelson, '23; Sure, '24) are shown in table 2. The figures represent the grams of each element in 4 gm. of salts, the amount usually included in 100 gm. of food. It is evident that these mixtures vary in the number and proportions of the elements included. Both the McCollum and Sure salts are made without iodine, but this is supplied in the drinking water. Of the four, only that of Osborne and Mendel contains added trace elements other than iodine, and none of them contains added copper.

These salt mixtures, particularly the Osborne and Mendel and the McCollum salts, are frequently used interchangeably, as in the United States Pharmacopoeia XI method for assaying vitamin A in cod liver oils. Such variations in the composition of a food as are produced when one of these mixtures is substituted for another, may not represent serious uncontrolled variables in some types of experiments. However, in any work involving the metabolism of inorganic salts, it is important to ascertain whether such differences as are indicated in table 2 may or may not be disregarded. This is especially necessary when dealing with the greatly enhanced rates of growth that have been observed in albino rats in

recent years (Osborne and Mendel, '26; Mendel and Hubbell, '35).

With regard to the change in rate of growth of this laboratory animal, it is interesting to note that Osborne and Mendel stated in 1911, "within one month a 70-gram white rat ordinarily will double its weight when the diet is adequate." So small a rate of gain is regarded as decidedly subnormal in

TABLE 2

Calculated composition of four common salt mixtures

	OSBORNE-MENDEL [1] (17)	MCCOLLUM [2] (20)	STEENBOCK (21)	SURE [3] (22)
	gm.	*gm.*	*gm.*	*gm.*
Calcium	0.462	0.276	0.296	0.322
Magnesium	0.0597	0.0581	0.0398	0.0681
Sodium	0.128	0.136	0.226	0.159
Potassium	0.684	0.463	0.513	0.541
Phosphorus	0.279	0.412	0.458	0.482
Chlorine	0.444	0.114	0.233	0.132
Sulfur	0.0259	0.0766	0.0525	0.0897
Iron	0.0112	0.0263	0.0101	0.0309
Iodine	0.000131		0.0020	
Manganese	0.000246			
Fluorine	0.000961			
Aluminum	0.0000219			

Data are in grams contained in 4 gm. of the mixture. the quantity usually included in 100 gm. of complete diet.

[1] Due to moisture these figures are lower than the theoretical values. They represent the composition of the salt mixture as used in these experiments.

[2] Iodine supplied in drinking water.

[3] First dsecribed by Steenbock as no. 32 salts. Iodine was supplied in the drinking water by Sure.

our colony today, and it seemed desirable to investigate the dietary requirements, in order to determine whether rations formerly considered adequate are suitable for an animal that gains 5 gm. a day instead of 2. The first step has been an examination of the inorganic constitutents of the diet, to ascertain to what extent some of the common salt mixtures fulfill the requirements for this more rapid development.

With this in view, the four salt mixtures described in table 2 were selected for detailed study. Inasmuch as a mixture may appear satisfactory when fed in an amount that assures liberal intake of the various ingredients, but may give rise to nutritive disturbances when the proportion in the diet is markedly increased or decreased, the four mixtures have been compared at various levels of intake. By decreasing the amount fed from the usual 4%, any lack or disproportion in the elements may become more evident. On the other hand, harmful effects due to an excess of any of the constituents may become sufficiently obvious to be noted if the amount fed is increased considerably above that ordinarily used. Consequently all four mixtures have been supplied at the following levels: 0.5, 1, 2, 3, 4, 5, 10 and 15% of the ration.

The bones are the largest storehouse for minerals in the body, and may be expected to reflect changes in the salt intake, particularly with respect to calcium and phosphorus. We have chosen, therefore, to evaluate the efficiency of the various mixtures not only by the rate of change in body weight during a selected period of active growth (60 to 200 gm.) but also by the composition of the bones. Preliminary experiments had indicated that the ash of the dry fat-free femurs should approximate 58 to 60% in the case of rats that make a daily gain of 5 gm. or more during the period specified.

The experimental procedure was the same for all groups studied. Male rats were weaned at 21 days of age, at an average body weight of 45 gm., and were placed in groups of ten on diets that contained casein 18, butter fat 9, lard 20, and dried yeast 8%. The remaining 45% of the ration consisted of starch and the salt mixture to be investigated; 10 drops of cod liver oil were supplied as a daily supplement, and distilled water was always available. Records were kept of food consumption, but no attempt was made to control the amount eaten. All animals were killed by ether at 200 gm. body weight and the paired femora were analyzed.

METHODS OF ANALYSIS

The femora from each animal were freed from tissue and dried at 105 to 107° for 4 days. The dry bones were then cracked in a mortar, transferred to a weighed ignited Gooch crucible and extracted for 2 days with 95% alcohol, and then for 2 days with ether. The extracted bones were dried at 105 to 107° for 6 to 8 hours and the weight of the 'fats' was obtained. The fat-free bones were then charred over a low flame and heated overnight in an electric muffle at about 600°, and the percentage of ash in the dry fat-free bones was calculated.

DISCUSSION

The data are summarized in table 3. In making comparisons of one salt mixture with another, it has been necessary to consider variations in the rate of growth within any one group. It has not been possible in this paper to include data for individual animals, but the ranges, both for the rate of gain per day and for the ash in the dry fat-free bones are given. Of the ten rats that received a diet containing 3% Osborne and Mendel salt mixture, one grew at a rate of 4.5 gm. a day and another at the rate of 6.6 gm. a day. The bone ash for the former was 59.9% in contrast to 56.1% for the latter. This difference in the mineral matter of the bones was accompanied by a corresponding difference in food intake. The more slowly growing rat consumed 350 gm. of food while the other ate but 278 gm. Similar differences in food intake help to explain the variations in bone ash in other groups.

At the level of salts most commonly used, 4% of the ration, the average rate of growth was the same for the Osborne and Mendel and for the McCollum salts, namely 5.2 gm. a day for the gain from 60 to 200 gm. body weight. The percentage of ash in the dry, fat-free bones was somewhat higher in the former group, however, being 60.8% in contrast to 57.9%. Both values, however, were of the desired magnitude for this rate of growth. For the animals on the Steenbock and the Sure salts, the bone ash figures were somewhat higher than for those fed the McCollum salts. This was accompanied by a

TABLE 3

Comparison of the effect of salt mixtures supplied at different levels in the diet [1]

SALT MIXTURE IN DIET	DAILY GAIN		TOTAL FOOD INTAKE, AVERAGE	TOTAL Ca INTAKE,[2] AVERAGE	TOTAL P INTAKE,[2] AVERAGE	ASH IN DRY FAT-FREE FEMURS	
	Range	Average				Range	Average
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	%	%
1%							
Osborne-Mendel	2.9–4.5	3.6	372	0.51	1.17	44.4–48.8	46.5
McCollum	2.3–4.0	2.8	439	0.40	1.52	42.9–49.1	46.2
Steenbock	2.4–4.0	3.0	436	0.41	1.57	42.2–49.4	46.2
Sure	2.5–4.7	3.0	454	0.45	1.66	42.1–51.6	46.5
2%							
Osborne-Mendel	5.2–6.4	5.8	298	0.75	1.15	49.7–53.2	51.8
McCollum	3.9–5.8	4.5	336	0.54	1.51	48.2–51.6	50.1
Steenbock	3.2–7.0	4.0	363	0.61	1.72	45.9–53.4	50.1
Sure	4.0–6.1	5.0	316	0.57	1.53	48.6–51.2	50.0
3%							
Osborne-Mendel	4.5–6.6	5.4	308	1.13	1.40	56.1–59.9	57.5
McCollum	4.2–6.1	4.8	330	0.76	1.82	52.8–57.6	55.5
Steenbock	3.4–6.4	4.5	325	0.79	1.91	52.9–57.2	55.4
Sure	3.5–7.0	5.0	304	0.79	1.84	50.9–59.3	54.4
4%							
Osborne-Mendel	4.2–6.4	5.2	324	1.57	1.70	59.5–62.4	60.8
McCollum	3.8–7.7	5.2	309	0.93	2.02	56.3–60.0	57.9
Steenbock	4.0–5.2	4.7	329	1.04	2.31	57.9–60.5	59.2
Sure	2.8–6.4	5.0	306	1.04	2.22	57.4–60.9	59.0
5%							
Osborne-Mendel	4.5–6.6	5.4	309	1.85	1.84	57.5–61.0	59.9
McCollum	4.4–5.6	4.8	323	1.20	2.44	58.1–61.6	60.3
Steenbock	4.4–6.4	5.4	295	1.15	2.41	57.1–60.6	58.7
Sure	3.8–5.8	4.7	337	1.42	2.85	57.2–61.8	59.9
10%							
Osborne-Mendel	4.7–6.1	5.6	308	3.63	2.91	58.8–62.2	61.1
McCollum	2.9–5.4	4.2	340[3]	2.45[3]	4.30[3]	59.6–62.8	61.3
Steenbock	3.4–5.2	4.1	356[3]	2.71[3]	4.95[3]	60.1–62.5	61.4
Sure	2.3–5.2	3.4	441	3.62	6.37	60.1–64.6	62.5
15%							
Osborne-Mendel	2.7–4.8	4.0	361[3]	6.35[3]	4.68[3]	60.7–65.0	63.0
McCollum	2.1–5.2	3.2	454[3]	4.86[3]	8.06[3]	59.6–65.0	62.4
Sure	2.8–3.2	3.0	413[3]	5.04[3]	8.45[3]	62.5–63.7	63.2

[1] Ten rats were used in each group except that with 15% Sure salts. There were five in this group.

[2] In all cases 100 gm. of food without the addition of salts supplied 0.020 gm. Ca and 0.245 gm. P.

[3] Food spilled and figures are estimates.

slightly lower growth rate in both cases. As the amount of salt mixture in the ration was decreased to 2 or 3%, the differences were still apparent as long as the rate of growth was maintained at approximately 5 gm. a day. In all cases, there appeared to be more complete calcification with the salt mixture that contained the higher proportion of calcium. When the salt intake was reduced to 1% of the ration, growth was so slow, an average of 3.1 gm. a day for all mixtures, that the differences in degree of calcification disappeared. The results obtained when each salt mixture was used as 0.5% of the diet have not been included in the summary because so few animals reached the required weight of 200 gm. It is perhaps significant, however, that eight out of a possible ten in the group which received 0.5% Osborne and Mendel salt mixture did reach 200 gm. Growth was slow, being but 2.6 gm. a day, and the bone ash was 40.9%, which is below the average figure for 21-day-old rats (42.8%). In addition, there were many signs of disturbed nutrition, such as diarrhoea, scattering of food, loss of hair, distorted bones, humped backs, etc. With the other salt mixtures, these disturbances were much more marked and there were also many cases of bloody eyes, sore legs, legs partly paralyzed, and general irritability. Several of the animals died before they reached 100 gm. in weight, and most of the others were killed when it became obvious that it would take so long for them to reach 200 gm. that comparisons with more rapidly growing rats would not be significant. When the McCollum salt mixture was used at this level (0.5%), one rat reached 200 gm. in 57 days, or at the rate of 2.4 gm. a day and the bone ash was 34.0%. Four animals fed the diet that contained 0.5% Sure salts completed their required gain in 73 days (1.9 gm. a day), and the bone ash was 43.0%. Although they grew at that greatly reduced rate, the animals were just able to maintain the amount of ash present in the bones at weaning.

When the amount of inorganic salts included in the ration was raised above the usual 4% level, it was evident that there was enough of all constituents present to insure adequate

calcification with all salt mixtures, independently of the rate of growth.

Attention should be called to the fact that, at the levels of 2 and 3%, growth compares quite favorably with that obtained when the larger amounts were used. It is thus evident that a lowering of the total amount of salts has no harmful effect on growth rate, but at these lower levels there is in no case sufficient calcium to ensure adequate bone formation for the rate of growth maintained.

The fact that satisfactory growth could be obtained with a total intake of 3 or even with 2% of salts, suggested that it

TABLE 4

Effect of increasing the calcium content of salt mixtures[1]

SALT MIXTURE IN DIET[2]	TOTAL Ca IN SALTS	AVERAGE DAILY GAIN	TOTAL FOOD INTAKE	TOTAL Ca INTAKE[3]	TOTAL P INTAKE[3]	ASH IN DRY FAT-FREE FEMURS
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	%
Osborne-Mendel + $CaCO_3$	0.58	5.8	288	1.73	0.91	59.5
McCollum + $CaCO_3$	0.58	5.8	318	1.91	1.11	58.5
Steenbock + $CaCO_3$	0.58	5.6	315	1.89	1.13	57.7
Sure + $CaCO_3$	0.58	5.0	334	2.00	1.22	58.9

[1] Ten rats were used in each group.

[2] In each case 1 gm. of the salt mixture was supplemented with sufficient calcium carbonate to furnish an amount of calcium equal to that in 5 gm. of Osborne and Mendel salts.

[3] In all cases 100 gm. of food without the addition of salts supplied 0.020 gm. Ca and 0.245 gm. P.

might be possible to prepare a salt mixture that would give the desired calcification when used in these smaller proportions. Consequently, to study the limiting factor or factors which prevent adequate calcification when the salt mixtures are used at low levels, a series of experiments was conducted in which various supplements were added to the original preparations. Only one of these is included inasmuch as it has the most direct bearing on the composition of our new salt mixture. To 1% of each of the four mixtures, calcium carbonate was added in sufficient quantity to furnish an amount of calcium equal to that supplied when 5% of Osborne and Mendel salts

is included in the diet. With this additional calcium, growth was rapid (5.0 to 5.8 gm. daily), and calcification (57.4 to 58.9%) was comparable to that produced when the unsupplemented salt mixtures were supplied as 4% of the diet. Table 4 shows the close agreement obtained in all cases.

SUMMARY AND CONCLUSIONS

Four common salt mixtures, the Osborne and Mendel, McCollum's 185, Steenbock's 40, and Sure's modification of the Steenbock 32 salts have been compared. As judged by rate of growth from 60 to 200 gm., and percentage of ash in the dry fat-free bones, the four mixtures are not interchangeable at any of eight levels studied. In spite of more rapid growth, higher bone ash was in general attained with the Osborne and Mendel salt mixture. Evidence is presented to show that, if adequate calcium is supplied, many other constituents can probably be furnished in amounts considerably below those ordinarily used.

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A NEW SALT MIXTURE FOR USE IN EXPERIMENTAL DIETS [1]

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A comparison of four salt mixtures in common use, namely the Osborne and Mendel, McCollum's 185, Steenbock's 40, and a modification of the Steenbock 32 salts, was reported in the preceding paper ('37). As judged by the criteria used, daily gain in weight during a selected period of active growth, and percentage of ash in the dry, fat-free femurs, the mixtures are not interchangeable in the diet of the albino rat at the various levels studied. It was also demonstrated that with each salt mixture, greater uniformity in respect to rate of growth and proportion of bone ash could be attained if the relative amount of calcium was considerably increased. When this was done, the total salt intake could be reduced from 4 to 2%. The bone ash produced under these conditions was sufficiently high to indicate that it should be possible to construct a mixture that could be successfully used at this lower level. The experimental work on which such a salt mixture is based is described in the present paper. Inasmuch as the new preparation is designed for use as a substitute for the Osborne and Mendel salts, a comparison of these two mixtures has been made under various conditions.

[1] The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington.

[2] Part of the experimental work reported in this paper was completed after the death of Professor Mendel. Prof. Arthur H. Smith has generously advised us during much of the investigation, and it is with pleasure that we acknowledge his cooperation.

The general plan of supplementation described in the preceding paper was employed to determine a suitable amount of Osborne and Mendel salts to be used as a basis for the modified mixture. Three amounts of the salt mixture, 0.5, 1 and 2 gm. per 100 gm. of food, were supplemented with calcium carbonate in an attempt to ascertain the quantities of the other ions required when the relative proportion of calcium is markedly increased. In the case of 0.5% of Osborne and Mendel salts, enough calcium carbonate was added to furnish a quantity of calcium equivalent to that present in 2 gm. of the unaltered salt mixture; with both 1 and 2% levels, the added calcium was in sufficient quantity to produce mixtures equivalent in calcium content to 4 and to 5 gm. of Osborne and Mendel salts, respectively. In each case, comparisons were made with amounts of unsupplemented salt mixture of equal calcium content. The feeding procedure, methods of analysis, and criteria were as described in the preceding paper. The data are shown in table 1.

TABLE 1

Effect of adding calcium to various levels of Osborne and Mendel salt mixture[1]

SALT MIXTURE IN DIET	TOTAL Ca IN SALTS	DAILY GAIN	TOTAL FOOD INTAKE	TOTAL Ca INTAKE[2]	TOTAL P INTAKE[2]	ASH IN DRY FAT-FREE FEMURS
%	gm.	gm.	gm.	gm.	gm.	%
0.5	0.06	2.2	518[3]	0.41[3]	1.45[3]	40.3
0.5 + $CaCO_3$	0.23	3.8	360	0.90	1.01	52.1
2	0.23	5.8	298	0.75	1.15	51.8
1	0.12	3.6	372	0.51	1.17	46.5
1 + $CaCO_3$	0.46	5.2	330	1.60	1.04	59.2
2 + $CaCO_3$	0.46	5.2	301	1.46	1.16	58.5
4	0.46	5.2	324	1.57	1.70	60.8
1 + $CaCO_3$	0.58	5.8	288	1.73	0.91	59.5
2 + $CaCO_3$	0.58	4.7	333	2.00	1.28	61.0
5	0.58	5.4	309	1.85	1.84	59.9

[1] Ten rats were used in each group.

[2] In all cases 100 gm. of food without the addition of salts supplied 0.020 gm. Ca and 0.245 gm. P.

[3] An approximation, due to spilling.

In general, the bone ash obtained by the use of the supplemented salts was of the same order of magnitude as that observed when the unsupplemented Osborne and Mendel salt mixture of equal calcium content was used. When Osborne and Mendel salt mixture formed 0.5% of the diet, growth was markedly subnormal and calcification of the femora (40.3%) was below that of animals at weaning (42.8% at 21 days). With calcium added to the level in 2 gm. of Osborne and Mendel salts, there was a distinct acceleration in growth and an increase in the percentage of bone ash to the amount characteristic of that produced when 2% of the unaltered salt mixture was used. When either 1 or 2% Osborne and Mendel salts was supplemented with calcium carbonate as described, the same trend was observed, and in addition, the bone ash was within the limits desired in a rat of 200 gm. body weight and approximately 50 days of age, namely 58 to 60%. It is evident from the comparisons recorded in table 1 that any of the combinations that supply as much calcium as 4 or 5% Osborne and Mendel salts should be satisfactory. The new salt mixture has, therefore, been devised on this basis and the other constituents are furnished in the amounts present in 1 to 2 gm. of the same mixture. As a measure of safety, the trace elements, including iron, have been supplied at a level approximately that in 4% Osborne and Mendel salts. Although there is enough copper present as a contaminant in most diets, this element has been included in the new mixture in order to provide for studies in which a high degree of purification of the ingredients of the diet might remove too large an amount of copper. The composition of the new salt mixture is given in table 2.

To prepare the salt mixture the first seven salts are pulverized and mixed thoroughly. Suitable quantities of separately prepared standard solutions of the remaining constituents are then added and, after a thorough mixing of all ingredients, the product is dried in an air drier for 6 to 8 hours at 80 to 85° and then ground in a mill to pass a 50-mesh sieve. It is possible, however, to make a satisfactory product without the

addition of aqueous solutions and the subsequent drying. The calculated composition of salt mixture 351 and of Osborne and Mendel salts is given in table 3. In the experiments that follow the new mixture has been used without the added copper, inasmuch as none is supplied in the original Osborne and Mendel salts.

TABLE 2

Salt mixture 351

	gm.		gm.
$CaCO_3$	543.0	$FePO_4.4H_2O$	20.5
$MgCO_3$	25.0	KI	0.08
$MgSO_4$	16.0	$MnSO_4$	0.35
NaCl	69.0	NaF	1.00
KCl	112.0	$Al_2(SO_4)_3K_2SO_4$	0.17
KH_2PO_4	212.0	$CuSO_4$	0.90

Figures are for 'reagent' or 'c.p.' salts.

TABLE 3

Calculated composition of salt mixtures

	351 [1]	OSBORNE-MENDEL [2]		351 [1]	OSBORNE-MENDEL [2]
Calcium	0.435	0.462	Iron	0.0103	0.0112
Magnesium	0.0209	0.0597	Iodine	0.000122	0.000131
Sodium	0.0554	0.128	Manganese	0.000254	0.000246
Potassium	0.239	0.684	Fluorine	0.000904	0.000961
Phosphorus	0.102	0.279	Aluminum	0.0000194	0.0000219
Chlorine	0.191	0.444	Copper	0.000716	
Sulfur	0.00908	0.0259			

Data are in grams contained in 2 gm. 351 salt mixture and in 4 gm. Osborne and Mendel salt mixture, the quantities usually included in 100 gm. of complete diet.

[1] Figures are for the salt mixture dried at 80 to 85° for 6 to 8 hours.

[2] Due to moisture these figures are lower than the theoretical values. They represent the composition of the salt mixture as used in these experiments.

The new mixture has been in use in this laboratory for about 2 years and has been compared with the Osborne and Mendel salt mixture under various experimental conditions. In order to subject the new preparation to a severe test, these comparisons were made when only 2% was incorporated in the food. Reference to table 3 shows that if this amount is supplied, the calcium content of the food is somewhat below

that with 4% Osborne and Mendel salts. There are some indications that 2% 351 salts may be near the lower limit of safety, particularly for short time experiments with very rapidly growing animals, and it is suggested that the amount be increased to 2.5% in such cases.

For the comparisons of the two salts, the food combinations summarized in table 4 were used. The diets were modifications of the mixture of purified foodstuffs described in the preceding paper. In all cases, except where noted, the general conduct of each experiment was as previously described.

TABLE 4

Percentage composition of diets[1]

DIET NO.	1	2	3	4	5	6	7	8	9[2]	10[2]
Casein	18	12	50	18	18	18				18
Lactalbumin							18	1.5		
Gliadin									18	
Butter fat	9	9	9	9		9	9	9	9	9
Lard	20	20	20	20		54	20	20	20	20
Starch	43	49	11		72	9	42–43	59.5	51	51
Dextrin				43						
351 salts	2	2	2	2	2	2	2–3	2	2	2
Yeast	8	8	8	8	8	8	8	8		

[1] Control diets for all series contained 4% Osborne and Mendel salt mixture with a corresponding reduction in carbohydrate.

Animals in all except series 5 received 10 drops of cod liver oil daily. Those in series 5 received 2 drops per week of a cod liver oil concentrate.

[2] Animals in series 9 and 10 received 400 mg. of yeast daily.

Table 5 presents data obtained with 12, 18 or 50% casein in the diet, 18% lactalbumin, a diet that contained no added fat other than that incidental to the fat-soluble vitamins employed, and one in which dextrin was used in place of starch.

The first comparisons, those of the animals fed 18% casein diets, represent larger numbers of animals than any of the other groups, inasmuch as positive controls of this type have been maintained at all times, in order to detect possible variations in the animals of the colony. By this means, every experiment has been controlled by the use of the standard

diet and with both salt mixtures. The average bone ash for the fifty-two control animals fed 2% 351 salts falls somewhat below that for the thirty-nine fed 4% Osborne and Mendel salt mixture, being 58.6% in contrast to 60.2%. This is due in part, however, to a difference in rate of growth, the average

TABLE 5

Comparison of salt mixture 351 and Osborne and Mendel salt mixture under different dietary conditions[1]

DIET NO.	TYPE OF DIET	SALTS IN DIET		DAILY GAIN	TOTAL FOOD INTAKE	TOTAL Ca INTAKE	TOTAL P INTAKE	ASH OF DRY FAT-FREE FEMURS
		Type	Per cent					
				gm.	*gm.*	*gm.*	*gm.*	%
1	18% casein	351	2	5.4	295	1.34	1.02	58.6
		351	2.5	5.4	286	1.64	1.07	60.2
		O–M	4	4.8	322	1.56	1.69	60.2
2	12% casein	351	2	5.0	355	1.62	0.94	59.6
		O–M	4	4.8	360	1.74	1.60	60.3
3	50% casein	351	2	5.0	283	1.39	2.21	57.9
		O–M	4	5.2	269	1.40	2.58	58.7
4	Dextrin	351	2	6.1	262	1.19	0.91	57.5
		O–M	4	5.4	277	1.34	1.45	58.1
5	Low fat	351	2	5.4	409	1.86	1.42	59.7
		O–M	4	5.8	385	1.86	2.02	59.4
7	18% lactalbumin	351	2	4.5	321	1.40	0.33	56.8
		351	3	5.3	284	1.85	0.43	58.1
		O–M	4	5.0	297	1.38	0.83	60.0

[1] With the control diet (18% casein) fifty-two rats were fed 2% 351 salts and thirty-nine rats were fed 4% Osborne and Mendel salts. Ten rats were used in each of the other groups.

for the animals of the first series being 5.4 gm. a day in contrast to 4.8 [3] gm. a day for the second. The average total food intake was considerably lower than 351 salts were used. With 2.5% of the new salt mixture, growth was at the same rate as with 2%, and the bone ash, 60.2%, was equal to that produced with 4% Osborne and Mendel salts.

[3] The figure for rate of growth, 4.8 gm. a day, is lower than that recorded in the preceding paper. It is an average of thirty-nine rats instead of the ten previously reported.

That the lower average bone ash for the animals fed 2% 351 salts is due in part to more rapid growth and the accompanying lowered food intake is seen in the results of a carefully controlled study of two groups of animals, in which the factor of variable food intake was eliminated. To make this comparison, litter mates of the same weight were selected and, in pairs, were fed the standard ration with either 2% 351 salts or 4% Osborne and Mendel salts. Unlike the usual procedure in paired feeding experiments, each animal was allowed to consume food ad libitum. Comparisons were made on the basis of equal food intake, the amount for a given pair being determined by that consumed by the first member of the pair to reach 200 gm. body weight. The second animal was killed when it had eaten the same amount of food. Of fourteen such pairs, three were discarded because the final weights were too divergent for satisfactory comparisons. The data are given in table 6.

Under the conditions of this experiment, there is very close agreement in bone ash for the two members of each pair. Although the average bone ash with salt mixture 351 as reported in table 5 is lower than when the Osborne and Mendel salt mixture was used, with these paired rats of the same initial and final weight and same food intake this difference is not observed. In spite of wide variations in rate of growth and bone ash for each salt mixture, the averages are the same, a gain of 5.3 gm. a day and a bone ash of 58.4%. The average total food intake of 290 gm. is of the same order of magnitude as that for the fifty-two control rats on 2% salt mixture 351 (table 5) and supplies further evidence that the slightly higher bone ash of rats fed Osborne and Mendel salts is due in part to an increased food intake.

It is not the purpose of this report to compare one ration with another, but merely to determine whether the new salt mixture can replace the old under various dietary conditions. On this basis we find comparable results in most cases. With some of the less common diets, such as that containing 12% casein, that with 50% casein, and the low fat diet, we find the

general type of results that would be anticipated. With both the 12% casein and the low fat diets there was greatly increased food consumption, and in both series the bone ash was approximately 60% with each salt mixture. With 351 salts

TABLE 6

Comparison of salt mixture 351 and Osborne and Mendel salt mixture under conditions of equal food intake

RAT NO.	SALTS IN DIET		DAILY GAIN	FINAL WEIGHT	TOTAL FOOD INTAKE	TOTAL Ca INTAKE	TOTAL P INTAKE	ASH OF DRY FAT-FREE FEMURS
	Type	Per cent						
			gm.	*gm.*	*gm.*	*gm.*	*gm.*	%
C7824	351	2	5.0	194	277	1.26	0.96	59.5
C7825	0–M	4	6.6	200	277	1.34	1.45	58.3
C7832	351	2	5.0	200	302	1.37	1.05	59.6
C7833	0–M	4	5.0	194	302	1.46	1.59	59.2
C7830	351	2	5.6	200	283	1.29	0.98	57.8
C7831	0–M	4	5.0	194	283	1.37	1.49	57.6
C7933	351	2	5.0	200	280	1.27	0.97	59.3
C7930	0–M	4	4.8	194	280	1.36	1.47	59.2
C7943	351	2	5.4	200	310	1.41	1.08	59.8
C7942	0–M	4	5.0	196	310	1.50	1.63	59.6
C8039	351	2	6.1	200	275	1.24	0.94	56.1
C8038	0–M	4	6.6	200	275	1.32	1.43	57.6
C8040	351	2	5.8	200	277	1.26	0.96	58.8
C8041	0–M	4	5.6	200	277	1.34	1.45	57.0
C8046	351	2	5.0	200	301	1.36	1.04	61.0
C8045	0–M	4	4.7	190	301	1.45	1.58	60.6
C8127	351	2	6.1	200	281	1.27	0.97	56.3
C8128	0–M	4	4.4	192	281	1.36	1.47	57.0
C8136	351	2	5.6	200	295	1.34	1.02	59.3
C8137	0–M	4	5.4	196	295	1.43	1.55	57.1
C8153	351	2	4.1	200	322	1.47	1.12	60.5
C8151	0–M	4	5.0	200	322	1.56	1.69	59.6
Average	351	2	5.3	199	290	1.32	1.01	58.9
Average	0–M	4	5.3	196	290	1.41	1.53	58.4

and 12% casein, growth was slightly below that of the control animals; with Osborne and Mendel salts there was no change in the growth rate as a result of the lower intake of protein. With the low fat diet and 351 salts, growth was normal (5.3

gm. a day) and with Osborne and Mendel salts it was decidedly above normal (5.8 gm. a day in contrast to 4.8 for the control diet). The intake of calcium and phosphorus was high in both cases and consequently good bone was produced in spite of the rapid growth. If the casein content of the diet was increased to 50%, food intake was low with both salt mixtures and the agreement in degree of calcification was good.

The experiment with lactalbumin as the protein was designed particularly to test the adequacy of the phosphorus supply in salt mixture 351. Although most rations in which a salt mixture is used would be likely to supply additional phosphorus, usually in the form of casein, it seemed desirable to determine what type of growth and what sort of bone would be produced when the only source of phosphorus was that found in the salt mixture itself. The mixture was supplied at two levels, the usual 2% and also at 3%. With 2% salts, growth was slow, being only 4.5 gm. a day, and the bone ash was significantly below that obtained when 4% Osborne and Mendel salts was used, being 56.8% in contrast to 60.0%; when the proportion of salt mixture was increased to 3% of the diet, growth was more rapid, being 5.3 gm. a day, and the bone ash was 58.1%. This figure for bone ash is still somewhat lower than that obtained with 4% Osborne and Mendel salts and lactalbumin as the protein. However, it is comparable with that produced when 2% 351 salts is used with an 18% casein food, and the rate of growth is practically the same. On the basis of this observation, it is suggested that 3% 351 salts be used with rations that supply no other phosphorus than that in the salt mixture.

Dextrin is frequently used to replace starch as a source of carbohydrate. When this substitution was made growth was rapid with low food intake and the bone ash was slightly below the desired 58 to 60% with both salt mixtures.

Although the main object of the experiments summarized in table 5 has been to demonstrate the adequacy of the new mixture as a substitute for the old, the comparisons that have been made have, in addition, furnished data concerning the

calcium and phosphorus intake of animals during the period of active growth from 60 to 200 gm. body weight.

The total and daily average intakes of calcium and phosphorus have been calculated for those groups in which the average rate of gain was in excess of 5 gm. a day and the bone ash was above 58%. With salt mixture 351, the average daily calcium intake varied from 44 to 59 mg., and the phosphorus from 13 to 45 mg. In the case of the Osborne and Mendel salts, the calcium intake was 42 to 64 mg. and the phosphorus 26 to 80 mg. per day. It is obvious that the intake of phosphorus has varied within wide limits without affecting the rate of growth or percentage of bone ash.

The individual records of the animals in the two control groups, namely those with either 2% 351 salts or 4% Osborne and Mendel salts in a diet containing 18% casein, have been studied in detail. With the new salt mixture, 75% of the animals gained in excess of 5 gm. a day on an average daily intake of 47 mg. calcium and 36 mg. phosphorus. With the Osborne and Mendel salt mixture, 54% of the rats gained at the same rate and the average daily calcium and phosphorus intakes were 55 and 60 mg., respectively. Inasmuch as an increase in the amount of 351 salts in the diet from 2% to 2.5% furnished very little more phosphorus and permitted the production of 60.2% bone ash, with no decrease in rate of growth (5.4 gm. a day), it would seem that an average phosphorus intake of 60 mg. per day was unnecessarily high under the conditions of this experiment.

Many nutrition investigations involve a limitation in the rate of growth by artificial means, or so-called 'stunting.' The two salt mixtures have accordingly been compared under certain of these conditions. These experiments, summarized in table 7, differ from those already described in that the rations were supplied for definite periods of time, since, under the conditions of feeding, the animals could not be expected to reach 200 gm. at a rate that would make comparisons significant. In the first series, the 'calorie control,' in which the standard rations were fed in suboptimal amounts, the period

of growth was 30 days for one group and 60 for another. The object of the longer period was to determine whether, with the low daily intake of calcium and phosphorus imposed by the restricted amount of food, there would be adequate calcification if the food was supplied over a sufficiently long period of time. Comparison of the two series shows that this is true, and also that differences in calcification, evident

TABLE 7

Comparison of salt mixture 351 and Osborne and Mendel salt mixture when growth is restricted[1]

DIET NO.	TYPE OF DIET	SALTS IN DIET		TIME ON DIET	FINAL WEIGHT	DAILY GAIN	TOTAL FOOD INTAKE	TOTAL Ca INTAKE	TOTAL P INTAKE	ASH OF DRY FAT-FREE FEMURS
		Type	Per cent	*days*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	%
1	Calorie control	351	2	30	111	2.1	150	0.68	0.52	54.6
		0–M	4	30	111	2.2	153	0.74	0.80	56.0
1	Calorie control	351	2	60	158	1.8	300	1.37	1.04	59.2
		0–M	4	60	155	1.8	306	1.48	1.61	59.9
8	1.5% lactalbumin	351	2	40	73	0.6	284	1.24	0.29	59.0
		0–M	4	40	74	0.7	245	1.14	0.69	59.8
6	High fat	351	2	30	127	2.7	183	0.83	0.64	53.7
		0–M	4	30	118	2.4	173	0.84	0.91	55.3
9	18% gliadin	351	2	30	68	0.6	131	0.60	0.13	57.6
		0–M	4	30	67	0.6	122	0.59	0.34	57.6
9	18% gliadin	351	2	30	71	0.8	150	0.68	0.15	
10	18% casein			30	200	4.3	290	1.32	1.01	62.1
9	18% gliadin	0–M	4	30	70	0.7	144	0.70	0.40	
10	18% casein			33	200	4.1	287	1.39	1.51	63.2

[1] In each of three groups, the 30-day calorie control, 1.5% lactalbumin, and the high fat, ten rats were used. Five rats were used in each of the other groups.

at 30 days, have almost disappeared at 60. For the 30-day period, the total intake of calcium is low, 680 mg. with salt mixture 351 and 740 mg. with Osborne and Mendel salts, yet with the slow growth characteristic of animals on a restricted food intake, calcification is comparatively good. Animals permitted to reach the same body weight when the rations were supplied ad libitum showed slightly lower bone ash with

both salt mixtures, namely 52.9 and 54.7% for the 351 salts and the Osborne and Mendel salts, respectively, as compared with 54.7 and 56.0% for the slowly growing animals with restricted food intake.

When an adequate protein was supplied in very small amount, 1.5% lactalbumin, for a period of 40 days after weaning, growth was almost negligible and the animals did not even double their weight in that time. The total intake of calcium and phosphorus was low, but evidently sufficient for the small bones of the stunted animals, for the percentage of ash is high with both salt mixtures. The bone ash for the much younger control rats of the same body weight is between 46 and 47% in contrast to 59% or over when growth was restricted by this means.

If growth was limited by the use of a diet high in fat, the same type of result, both in rate of gain and percentage of bone ash, was obtained as when the intake of the standard ration was kept subnormal. Though the animals were permitted to consume food ad libitum, the intake was small, being only 10 to 15% above that consumed by the calorie restricted animals.

Retardation of growth due to the use of an incomplete protein, gliadin, resulted in a rate of gain that was very low. At the end of 30 days, calcification was relatively high for the body weight attained, being 57.6% for both salt mixtures in contrast to 47% for the controls at the same body weight. In another series a 30-day period of restricted growth with gliadin as the protein was followed by realimentation with a diet containing casein. The animals were permitted to reach the usual body weight of 200 gm. and calcification at the end of the total growing period of approximately 60 days was excellent in both cases.

SUMMARY

A new salt mixture has been described in which the proportion of calcium to other constituents has been greatly increased. This preparation, made by mixing dry salts, may be used at the level of 2% in many diets of purified foods which usually require 4% Osborne and Mendel salts.

In experiments designed to compare the two salt mixtures during a selected period of active growth, from 60 to 200 gm. body weight, at a rate in excess of 5 gm. a day, adequate calcification of the femora has resulted with an average daily intake of approximately 50 mg. calcium and 35 mg. phosphorus.

LITERATURE CITED

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THE EFFECT OF EXERCISE ON METABOLISM FOLLOWING THE INGESTION OF WATER, GLUCOSE AND FRUCTOSE, AS SHOWN BY THE COURSE OF THE RESPIRATORY QUOTIENT

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TWO FIGURES

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In a recent study (Bachmann and Haldi, '37) a rise in blood lactic acid and a fall in blood CO_2 were observed within 30 minutes after the ingestion of fructose, but not after glucose. Respiratory quotients after fructose, corrected for non-metabolic CO_2 in the expired air, in some instances remained above unity, indicating formation of fat; after glucose the quotients never reached unity. Since exercise increases the proportion of carbohydrate oxidized, these differences in the metabolism of glucose and of fructose raise several interesting questions: If glucose or fructose were ingested immediately before exercise, would a greater part of the energy of exercise be supplied by carbohydrate than when no sugar is taken? Would both sugars serve equally well to spare the carbohydrate stores of the body? Would exercise have an accelerating effect on the metabolism of either or both these sugars, with a greater utilization of the ingested sugar during exercise than at rest?

An attempt to answer these questions has been made in the present study of the respiratory exchange after the ingestion of glucose and of fructose, both with the subject at

rest and taking exercise immediately after ingestion of the sugar. Although a similar study was reported several years ago by Carpenter and Fox ('31 b, '31 c) we believed the question to be of sufficient importance to warrant further investigation.

METHOD

The respiratory exchange was determined by the open circuit method of Carpenter and Fox ('31 a). The gas meter was read at 15-minute intervals; the gas samples collected in the manner described eleswhere (Bachmann and Haldi, '37), were analyzed by two analysts using the Haldane-Henderson method. The duplicate analyses were required to check within 0.02%.

The subjects of the experiments were two male adults, one (J. H.) 38 years of age, 182 cm. in height, weighing 80 kilos, the other (W. W.) 27 years of age, 170 cm. in height, weighing 56 kilos. All the experiments were conducted at approximately the same time of day, from 7.30 to 11.00, the last meal having been taken not later than 7 o'clock the evening before. On the morning of the experiment the subject rode to the laboratory and then reclined for 30 minutes or more on a couch placed near the respiration apparatus.

After the preliminary rest the respiratory exchange was determined for three consecutive 15-minute post-absorptive or basal periods. The subject then arose, voided urine and ingested either 500 cc. of water alone at 37°C. or 50 gm. of glucose or fructose, or 25 gm. of each in the same amount of water. The control experiments with water alone were suggested by those of Carpenter and Fox ('30) which showed a measurable increase in metabolism after 500 cc. water. Two minutes after the beginning of the ingestion, a signal was given for the resumption of the experiment.

Two types of experiments were conducted: One, with the subject remaining at rest throughout seven 15-minute periods after ingestion; the other, with the subject taking exercise on a Prony brake bicycle ergometer for two consecutive 15-minute

periods immediately after the ingestion, and then reclining for five 15-minute periods. The exercise experiments were preceded by a 3 weeks period of training during which the same amount of work was done on 5 or 6 days a week as in each of the experiments.

In the rest experiments the subject reclined on the couch continuously except for 2 or 3 minutes at the end of the post-absorptive periods when he arose to void urine and ingest the water or sugar solution. In the exercise experiments, he mounted the bicycle immediately after the ingestion, inserted the mouthpiece and at a given signal began the exercise. The tension on the ergometer wheel was maintained constant at 2.5 kg. and the rate of pedalling timed to the beat of a metronome. Work was done at approximately 550 kilogrammeters per minute. Upon the completion of the exercise, the subject 'rolled off' the bicycle on to the couch without interrupting the experiment. Whenever it appeared from the kymograph record that the subject was about to go to sleep a buzzer was sounded, to which he responded with the same signal. At the end of the experiment the urine was again collected. The total nitrogen of the urine samples was determined for calculating the non-protein respiratory quotients.

RESULTS AND INTERPRETATION

For the sake of clarity a brief description of the respiratory quotients in the rest experiments will be given first, followed in order by a description and interpretation of the quotients of 1) the two exercise periods, 2) the first recovery period, 3) the last four recovery periods. The non-protein respiratory quotients hereafter referred to as the 'respiratory quotients,' are shown for the various groups of rest and exercise experiments in figure 1, each curve representing an average of at least three experiments. The average of all the post-absorptive quotients has been taken as a common base line. Figure 2 facilitates a comparison of the increment in the respiratory quotient during any period of the various groups of experiments. It will be observed from the curves

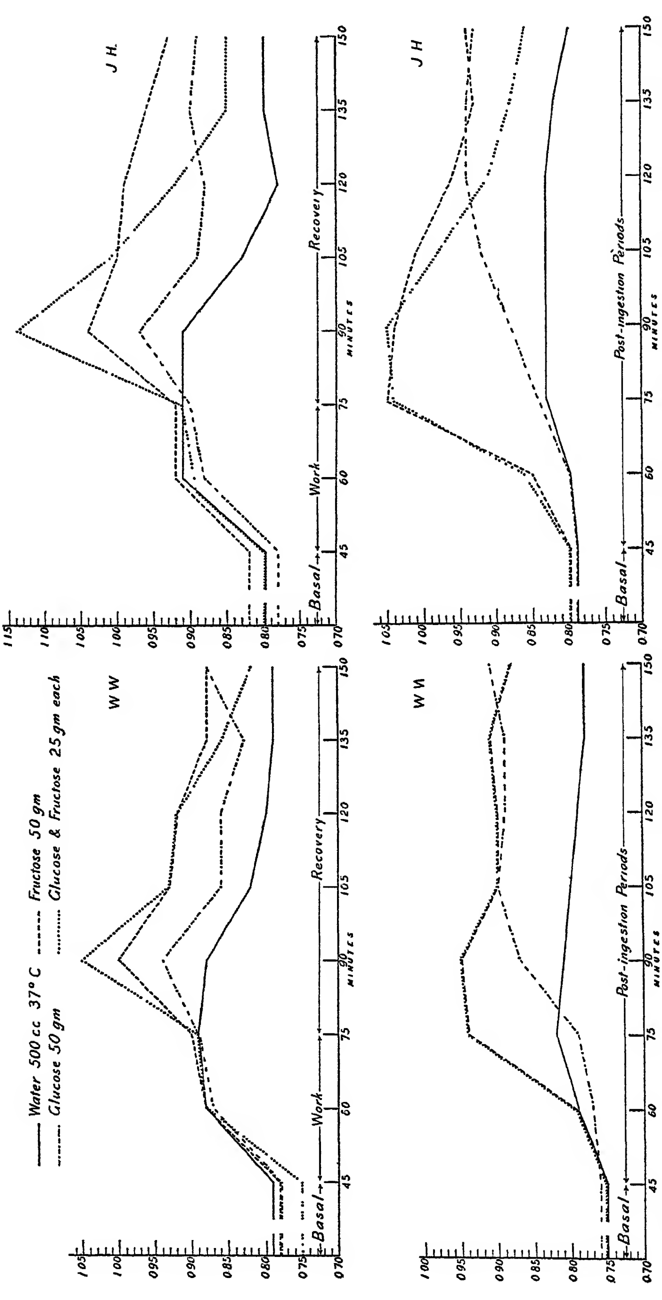

Fig. 1 The course of the respiratory quotient in two subjects (W. W. and J. H.) following the ingestion of water, glucose, fructose and a mixture of these two sugars when at rest (lower curves), and during exercise and recovery (upper curves).

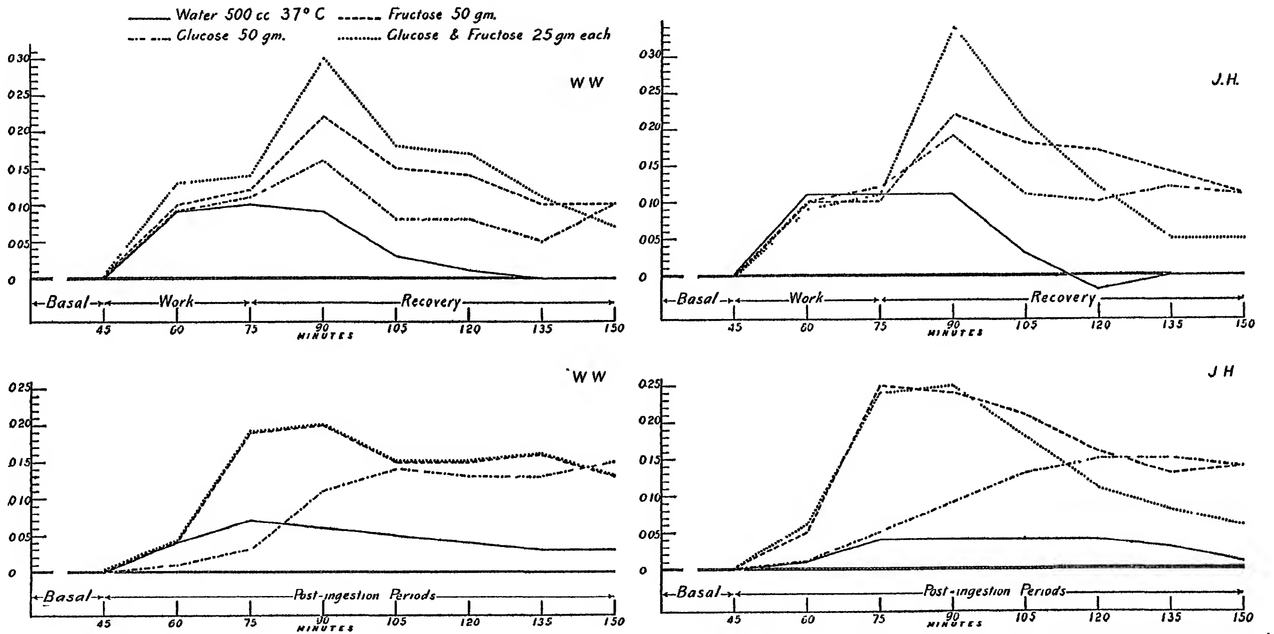

Fig. 2 Increments of the respiratory quotient above the base line in two subjects following the ingestion of water, glucose, fructose and a mixture of these two sugars when at rest (lower curves), and during exercise and recovery (upper curves).

in figures 1 and 2 that the qualitative results with both subjects are the same while the quantitative data show a fairly close approximation.

The respiratory quotients of the rest experiments. After the ingestion of water in the control experiments the respiratory quotients of the two subjects rose from the base line of 0.76 and 0.79 to 0.82 and 0.83, respectively, an increase of 0.06 and 0.04, and then gradually dropped toward the post-absorptive level. At the end of the experiment the quotient of each subject was still slightly above the base line.

In the glucose experiments the respiratory quotient remained practically stationary during the first 15-minute period after ingestion, and then in the next 15-minute period began a gradual ascent, reaching a maximum of 0.90 and 0.94 an increment of 0.14 and 0.15, respectively, with the two subjects, 45 and 60 minutes after ingestion, and continuing at approximately this level during the remaining 30 minutes of the experiment.

In the fructose experiments the respiratory quotient showed a distinct rise above the base line during the first 15-minute period after ingestion; in the next period the rise was more abrupt bringing the quotient almost to the maximum of 0.95 with one subject and 1.05 with the other, a respective increase of 0.20 and 0.25. In the fourth period there was a drop in the quotient of each subject and from this time on, the quotient of W. W. remained at practically the same level while that of J. H. continued in its descent toward the baseline. At the conclusion of the experiment, the respiratory quotients in the glucose and fructose experiments were approximately the same distance above the base line (fig. 1).

In the experiments with a mixture of the sugars the respiratory quotient of W. W. followed throughout that of the fructose experiments; the respiratory quotient of J. H. also followed that of fructose until 60 minutes after ingestion when it fell more abruptly and came closer to the base line at the end of the experiment. These observations agree with those of Deuel ('27) who obtained practically the same results

from 75 gm. fructose as from a 75 gm. mixture of fructose and glucose in equal parts.

The respiratory quotient of exercise. During the first 15-minute exercise period there was a marked rise in the respiratory quotient in all the experiments. With subject W. W. it attained approximately the same level in the water as in the sugar experiments, whereas there was some variation with subject J. H. The actual rise above the base line, however, as seen in figure 2 was approximately 0.10 in all the experiments except in those on W. W. with a mixture of the sugars. The respiratory quotient was usually slightly higher in the second than in the first 15-minute exercise period.

Interpretation. In the control experiments there was an increase in both the absolute and relative amount of carbohydrate oxidized. Whereas carbohydrate supplied only about 25% of the total energy during the post-absorptive periods, approximately 65% was derived from this foodstuff during exercise.

The percentage of carbohydrate oxidized as shown by the respiratory quotient was not increased by the ingestion of the sugars immediately before exercise. It is worthy of note that the ingested glucose and fructose were equally ineffective in this respect. The experiments on W. W. with a mixture of the sugars in figure 2 are somewhat irregular. As this irregularity did not occur in the absolute respiratory quotient (fig. 1), we believe that it should be attributed to an unusually low post-absorptive quotient which was obtained in these experiments and not to a specific effect of the mixture of the sugars. The average post-absorptive quotient was only 0.75 as contrasted with quotients of 0.79, 0.78 and 0.78 obtained in the water, glucose, and fructose experiments, respectively. We are at a loss to account for the unusually low quotient.

The total amount as well as the percentage of carbohydrate oxidized during exercise was unaffected by the previous ingestion of the sugars. The total amount metabolized by W. W. during the two 15-minute exercise periods was 29, 29, 29 and 30 gm. in the experiments with water, glucose, fructose

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The total amount as well as the percentage of carbohydrate oxidized during exercise was unaffected by the previous ingestion of the sugars. The total amount metabolized by W. W. during the two 15-minute exercise periods was 29, 29, 29 and 30 gm. in the experiments with water, glucose, fructose

and a mixture of the sugars, respectively, and by J. H. 32, 32, 33, and 33 gm., respectively. The average difference of 1 to 2 gm. for the ½ hour of exercise is regarded as of little or no significance, since the same variation was observed in individual experiments of the control series.

It is obvious from figure 2 that the rise in the respiratory quotients produced by the sugars themselves as shown in corresponding periods of the rest experiments, was not superposed upon the rise induced by exercise. On casual examination it may appear as if the metabolism of the ingested sugars was inhibited by exercise. This apparent inhibition is especially striking in the case of fructose and the mixture of glucose and fructose. It can be shown from analysis of the data, however, that such a conclusion is unjustified. In the experiments with fructose, for example, the changes in the oxygen consumption and carbon dioxide elimination were sufficient to produce a marked increase in the respiratory quotient in the rest experiments. They were so small, however, relative to the oxygen consumption and carbon dioxide elimination during exercise, that when added to the latter they had no appreciable effect on the respiratory quotient. It is reasonable to assume that the metabolism of the ingested sugars proceeded to at least as great an extent during exercise as in the corresponding periods of the rest experiments, thereby serving to spare the body stores of carbohydrate.

The respiratory quotient of the first 15-minute recovery period. The respiratory quotient during the first recovery period in the control experiments remained at practically the same level as during exercise. In the experiments with the sugars it rose well above the exercise level. The greatest rise occurred in the experiments with a mixture of the sugars and the smallest in the glucose experiments. With the mixture the quotient of each subject went above unity (fig. 1) while with fructose that of one subject rose to unity and that of the other above unity; with glucose the highest average quotient was 0.94 for one subject and 0.97 for the other.

Interpretation. The increase in carbohydrate metabolism induced by exercise as shown in the control experiments persisted for a few minutes after exercise. The interpretation of the first recovery period of the sugar experiments presents some difficulties; first, because of the marked rise of the quotients during this period above that of the exercise level, and secondly, because of the greater rise with the mixture of the sugars than with glucose or fructose alone. As regards the rise above the exercise level, the possibility that it was due to lactic acid formation with the consequent blowing off of non-metabolic carbon dioxide should first be considered, because of the increase in blood lactic acid observed after the ingestion of fructose (Campbell and Maltby, '28; Bachmann and Haldi, '37). There are reasons, however, militating against this possibility: 1) While there was no appreciable difference in the amount of lactic acid formed from 50 gm. fructose or a mixture of 25 gm. glucose and 25 gm. fructose, in the present experiments there was a considerably greater increase in the respiratory quotient of the first recovery period in the experiments with a mixture of the sugars than with fructose; 2) the large rise in the quotient during the first recovery period in the experiments with fructose or a mixture of the sugars, could not be accounted for by the liberation of non-metabolic carbon dioxide, even though it be assumed that the maximum amount of lactic acid found in the experiments quoted above, was formed in that period.

There is the possibility that the metabolism of the sugars may have been superposed upon the normal metabolism of recovery. A quantitative analysis has accordingly been made of the data on the oxygen consumption and carbon dioxide elimination of the first recovery period in the exercise experiments and of the corresponding period in the rest experiments. The increase in oxygen consumption and carbon dioxide elimination due to the sugars in the third period after ingestion in the rest experiments when added to the oxygen consumption and carbon dioxide elimination of the first recovery period in the control experiments (normal metabolism

of recovery) gave respiratory quotients lower than those actually obtained in the exercise experiments with the sugars. While the results are not conclusive, they are nevertheless highly suggestive. It would appear that the rise of the respiratory quotient above the exercise level in the first recovery period of the sugar experiments was due only in part to a superposition of the metabolism of the sugars upon the normal metabolism of recovery. The failure of the theoretical quotients to coincide more closely with the actual indicates that a change had been induced in the metabolism of the sugars during exercise which persisted during the first recovery period.

Further analysis of the data give suggestive results as regards the nature of this change. In the sugar experiments the average net excess oxygen consumed in the first recovery period was subtracted from the average net excess carbon dioxide produced to obtain what may be designated as the surplus carbon dioxide. The surplus carbon dioxide of the corresponding period of the rest experiments was obtained by the same procedure. Since in the oxidation of carbohydrate one volume of oxygen yields one volume of carbon dioxide, there should be no surplus carbon dioxide, if the excess oxygen were used exclusively for the oxidation of the ingested sugars. In the first recovery period of the exercise experiments with glucose, fructose and a mixture of the sugars, it was 250, 510 and 1000 cc., respectively, and in the corresponding periods of the rest experiments, 110, 480 and 430 cc., respectively. It will be observed that whereas the surplus carbon dioxide was the same in the rest and exercise experiments with fructose, it was greater in the exercise than in the rest experiments with glucose and a mixture of the sugars. These data suggest a transformation into fat of a part of the ingested glucose and fructose at rest and in the case of glucose an acceleration of the process during exercise, which continued during the first period of recovery. The acceleration may have been due perhaps to an increase in body temperature (compare Pembrey and Nicol, 1898). The larger

surplus carbon dioxide in the exercise experiments with a mixture of the sugars than with fructose would account for the greater rise in the respiratory quotient. An objection to this interpretation is found in the greater surplus of carbon dioxide in the experiments with a mixture of the sugars than in those with glucose. It is not at all impossible, however, that the metabolism of glucose under the influence of exercise may have been modified by the simultaneous metabolism of fructose.

The respiratory quotients of the last four recovery periods. In the control experiments the respiratory quotient of each subject fell abruptly during the second recovery period; in the next period, it declined practically to the base line where it remained to the end of the experiment.

In all the sugar experiments the respiratory quotient likewise fell abruptly during the second recovery period. From this time on the quotient remained almost constant in the glucose experiments, whereas it fell gradually toward the base line in those with fructose. In the experiments with a mixture of the sugars the fall was gradual with one subject and abrupt with the other, for three recovery periods; it then remained constant during the last 15-minute period. At the conclusion of the experiments the quotients were well above the base line in all the sugar experiments, being at the highest level with one subject with fructose, the lowest with a mixture of the sugars and at an intermediate position with glucose; with the other subject, the quotient of glucose ascended abruptly to the level of that of fructose. If this unusual rise had not occurred, it is likely that approximately the same relations would have prevailed as with the other subject. A quantitative comparison of the net increase in the respiratory quotients of the last four recovery periods with the corresponding periods of the rest experiments is presented in table 1. The net increase represents the difference between the increase above the post-absorptive level in the control and that in the sugar experiments.

Interpretation. The smaller net increase of the respiratory quotients in the exercise than in the rest experiments after glucose indicates that there was less of the ingested sugar

TABLE 1

Net increase in the respiratory quotient during the last four 15-minute recovery periods and in the corresponding periods of the rest experiments

SUGAR INGESTED	SUBJECT	TYPE OF EXPERIMENT	NET INCREASE [1]			
			(1)	(2)	(3)	(4)
50 gm. glucose	W. W.	Rest	0.09	0.09	0.10	0.12
		Exercise	0.05	0.07	0.05	0.10
	J. H.	Rest	0.09	0.11	0.12	0.13
		Exercise	0.08	0.08	0.12	0.11
50 gm. fructose	W. W.	Rest	0.10	0.11	0.13	0.10
		Exercise	0.12	0.13	0.10	0.10
	J. H.	Rest	0.17	0.12	0.10	0.13
		Exercise	0.15	0.15	0.14	0.11

[1] The net increase was obtained by subtracting the increase over the basal in the control experiments with water from the increase over the basal in the sugar experiments.

TABLE 2

Net increase in carbohydrate oxidized during the last four 15-minute recovery periods and in the corresponding periods of the rest experiments

SUGAR INGESTED	SUBJECT	TYPE OF EXPERIMENT	NET INCREASE,[1] GRAMS CARBOHYDRATE				
			(1)	(2)	(3)	(4)	Total
50 gm. glucose	W. W.	Rest	1.3	1.3	1.2	1.5	5.3
		Exercise	0.6	0.8	0.5	1.2	3.1
	J. H.	Rest	1.3	2.1	1.7	1.9	7.0
		Exercise	1.0	1.4	1.4	1.1	4.9
50 gm. fructose	W. W.	Rest	2.1	2.3	1.5	1.2	7.1
		Exercise	2.1	2.2	1.7	1.5	7.5
	J. H.	Rest	1.6	2.2	1.6	1.9	7.3
		Exercise	2.3	2.6	1.8	1.5	8.2

[1] The net increase was obtained by subtracting the increase over the basal in the control experiments with water from the increase over the basal in the sugar experiments.

available for metabolism during recovery than in the corresponding rest periods. It may therefore be concluded that the metabolism of the ingested glucose was accelerated by exercise.

In the fructose experiments there was no appreciable nor consistent difference between the net increase of the quotients in the exercise and rest experiments. The metabolism of the ingested fructose, therefore, was not accelerated during exercise. These observations on the two sugars agree with those of Carpenter and Fox ('31 c).

Calculations by the same procedure of the net increase in carbohydrate oxidized (table 2) show that in the glucose experiments the average amount of carbohydrate burned during the recovery periods was 2.2 gm. less than in the rest experiments. This confirms the conclusion drawn from the respiratory quotients that the metabolism of glucose was accelerated during exercise thereby reducing the amount available during recovery. In the experiments with fructose practically the same amount of carbohydrate was oxidized during recovery as in the corresponding time in the rest experiments.

DISCUSSION

The results of these experiments, as stated above, suggest a conversion of a portion of the ingested glucose into fat under resting conditions and an acceleration of this process by exercise. It would appear therefore as if the respiratory quotients obtained after the ingestion of glucose are not exclusively combustion quotients. The common assumption that the respiratory quotients after the ingestion of glucose are combustion quotients is probably based on the close agreement in heat production measured by direct and indirect calorimetry, as reported by Lusk ('15) following the ingestion of the sugar when dissolved in warm water. It should be noted, however, that in a subsequent study, Murlin and Lusk ('15) found in each of six experiments on the dog that the heat production after the administration of 70 gm. glucose was greater by indirect than by direct calorimetry. Cathcart and Markowitz ('27) from a statistical analysis of these data, found a significant difference between the heat production as determined by the two methods. While they recognized that the data are insufficient for satisfactory treatment by statistical methods, their analysis assumes greater significance in

view of the constancy with which a higher heat production was obtained by the indirect method. These results suggest that the respiratory quotients from which the heat production was calculated, were not exclusively combustion quotients.

The failure of most workers to obtain respiratory quotients above unity after the ingestion of glucose may be partly responsible for the assumption that the quotients are true combustion quotients. As aptly stated by Rapport ('30), a respiratory quotient of unity has been established as a sort of dead line which makes it seem as though the conversion into fat cannot take place unless this quotient is exceeded, although there is no theoretical reason why this should be the case. Benedict and Carpenter ('18) some years previously had also called attention to the inference frequently made that the formation of fat from carbohydrate can occur only when the respiratory quotient is above unity. These authors, like Rapport, recognized the fallacy of this assumption.

Positive evidence for the conversion of glucose into fat is found in some of the respiratory quotients reported in the literature. Benedict and Carpenter ('18) obtained one measured respiratory quotient of 1.01 for one 30-minute period and several quotients of 0.98 and over. Since the "non-protein respiratory quotients were generally two or three points higher than the measured quotients, all values of 0.98 or over would, strictly speaking, represent a non-protein respiratory quotient of unity." Tögel, Brezina and Durig ('13) obtained a quotient of 1.03 in man after the ingestion of 100 gm. of glucose and Hanriot (1892; cited by Rapport, '30) a quotient of 1.07 after the administration of 73 gm. of glucose. The last named author has also published much higher quotients after glucose, although their accuracy has been questioned. Lublin ('26) found that quotients of his non-diabetic subjects rose in some instances above unity after the ingestion of 100 gm. glucose. Respiratory quotients well above unity were obtained on the dog by Lusk ('15) after the administration of 50, 60 and 70 gm. glucose. Similar results were obtained by Murlin and Lusk ('15) and by Morgulis and Pratt ('13).

As regards the respiratory quotients after the ingestion of fructose, it was shown in a previous communication (Bachmann and Haldi, '37) that they are not exclusively combustion quotients. In view of this observation and the foregoing considerations with respect to glucose, it becomes obvious that calculations of the amount of carbohydrate metabolized, heat production and the specific dynamic action of the sugar, based on the respiratory quotient, would yield incorrect values. The error in heat production, however, would be small because of the slight change in the value of the caloric factor within the range of the possible error of the respiratory quotient.

Calculations of the amount of carbohydrate oxidized after the ingestion of the sugars in these experiments, as shown in table 2, were made nevertheless, since it is likely that an error of the same order occurred in both the rest and exercise experiments. The conclusions drawn from a comparison of the two sets of experiments are, therefore, probably substantially correct. It should be noted that these conclusions support those arrived at from a consideration of the course of the respiratory quotient.

The results of our experiments differ in some respects from those obtained by Carpenter and Fox ('31 b). During exercise of the same intensity and duration, the respiratory quotients of their subject rose considerably higher than those of our two subjects, in some instances exceeding unity. The average quotients for the two periods of work on their control experiments were 1.00 and 0.96, whereas the average in our experiments was 0.88 and 0.89 with one subject and 0.91 in both periods with the other subject. Our lower quotients may possibly be accounted for by a more robust physique of our subjects. The high quotients obtained by Carpenter and Fox, some of which were above unity, suggest an accumulation of lactic acid and hyperventilation during exercise. The drop in the quotient below the base line in the second recovery period and its gradual rise toward the base line throughout the remainder of the experiment, would therefore appear to have been due to a compensatory retention of carbon dioxide during

the liquidation of the oxygen debt. In our experiments the quotient fell gradually after the first recovery period until it reached the base line where it remained until the end of the experiment. Carpenter and Fox believe that the fall in quotient below the base line indicates that the effect of work was to exhaust the available carbohydrate. If this were the case, it is difficult to account for the gradual rise in the quotient toward the base line which they observed during recovery.

The rise in the respiratory quotients obtained in the first recovery period of our experiments with the sugars was not observed by Carpenter and Fox. This difference in results can be explained if we assume that hyperventilation occurred in their experiments for, although the quotient fell below the base line after exercise in their glucose and fructose experiments, it was nevertheless 0.07 and 0.10 higher than in their control experiments with water. Notwithstanding these differences in experimental results some of our conclusions are essentially in accord with theirs. It can be readily seen that conclusions based on the difference between the results obtained in their sugar and control experiments would not have been invalidated by the effects of hyperventilation if the hyperventilation were practically the same in all the experiments.

SUMMARY AND CONCLUSIONS

The respiratory quotient as affected by exercise taken immediately after the ingestion of 500 cc. water, 50 gm. glucose, 50 gm. fructose, and a mixture of 25 gm. each of these two sugars dissolved in 500 cc. water at 37°C. has been studied on two subjects. The exercise consisted of performing 550 kilogrammeters of work per minute for two consecutive 15-minute periods on a Prony brake bicycle ergometer.

In the control experiments with water, the respiratory quotient rose to approximately 0.90 during exercise, an increase of 0.10 above the base line. The quotient of one subject was slightly higher during the second 15-minute period, whereas that of the other remained the same.

The increase of the respiratory quotient during exercise was practically the same when the sugars were ingested as when water alone was taken immediately before the exercise.

The rise in the respiratory quotient during exercise in the experiments with water and the sugars shows that there was an increase in the relative and absolute amount of carbohydrate oxidized.

Since the same rise in the quotient was obtained in the experiments with the various sugars as in the control experiments with water, it is concluded that the percentage of carbohydrate oxidized was not increased by the ingestion of the sugars immediately before exercise. Glucose and fructose were equally ineffective in raising the percentage of carbohydrate oxidized during exercise.

Practically the same amount of carbohydrate was oxidized during exercise in the control and various sugar experiments. In all the experiments there was more carbohydrate oxidized in the second than in the first exercise period.

During the first recovery period in the control experiments with water the respiratory quotient remained at practically the same level as during exercise, indicating that the increase in carbohydrate metabolism induced by exercise persisted some few minutes afterward.

In the experiments with the sugars there was a rise in the quotient during the first recovery period above the level reached during exercise. The greatest rise occurred in the experiments with a mixture of the sugars, the least in those with glucose. This may be explained in part as a superposition of the metabolism of the sugars on the normal metabolism of recovery. The data have been interpreted as suggestive of the conversion of a portion of the glucose into fat and of an acceleration of this process by exercise.

Exercise accelerated the metabolism of glucose whereas it had no effect on that of fructose. Inasmuch as the ingested glucose did not increase the total carbohydrate oxidation during exercise, it must have had a greater sparing effect than fructose on the body stores of carbohydrate.

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VITAMIN E AND GROWTH[1]

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TWO FIGURES

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On several occasions it has been suggested that vitamin E promotes growth and general well-being in addition to preventing sterility in male and female rats.

Evans ('28) demonstrated an impairment in the late growth of animals reared on a vitamin E-deficient diet. At 150 to 200 days of age both male and female animals ceased to grow, while litter mate animals which had received or were then given wheat germ oil or sterol-free concentrates continued to grow normally or resumed growth. Since castrated males responded to the same treatment the stimulus to growth was not mediated through the gonads. The basal diet contained 22% lard and 2% cod liver oil.

Mason ('29) observed that lettuce induced a marked increase in early growth rate of male rats when added to a diet apparently deficient only in vitamin E. From his studies and from a review of the earlier work of others he concluded that vitamin E per se was not responsible for this growth stimulation.

More recently Blumberg ('35) reported a series of feeding trials in which animals on vitamin E-deficient diets ceased growing when they were younger and at much lower weight levels than those of Evans. The rations were fat-free except

[1] Presented in part before the thirtieth meeting of the American Society of Biological Chemists at Washington, March 28, 1936 (J. Biol. Chem., vol. 114, p. lxxvii, 1936).

for small amounts of ethyl linolate and of ethyl laurate and oleate used as solvents for carotene and irradiated ergosterol. The animals became lethargic and later almost helpless. Their miserable condition could be improved by providing daily 1 gm. of wheat germ or 2 gm. of egg yolk, less well by 10 gm. of fresh lettuce and doubtfully by butter fat (0.5 gm.) or pasteurized milk (25 cc.). The negative results secured with supplements of the other recognized accessories and the accepted presence of vitamin E in the successful supplements led to the conclusion that vitamin E or some unrecognized associated fat-soluble factor was required for normal early and middle growth as well as for late growth and fertility.

Since our experiments were first reported (Olcott and Mattill, '36), Emerson and Evans ('37),[2] using female rats, have completed experiments successfully duplicating Evans' results and conclude that "the defective growth in experiments involving vitamin E occurs only after the 4th month of life." They were thus not able to confirm Blumberg's results.

In the observations of Ringsted ('35) on mature animals which had been deprived of vitamin E from weaning, emphasis is laid upon a paresis of the hind quarters which appeared at 160 to 220 days of age and which was accompanied later by symmetrical denuding and a hypoaesthesia of legs and tail. Animals which had, for test purposes, received vitamin E in the form of wheat germ oil and in amounts adequate for a successful gestation, remained free from these symptoms; the animals which demonstrated them had been used for assaying other possible vitamin E containing foods, all of them giving negative results. Ringsted found the condition somewhat similar to that in weanling rats first described by Evans and Burr ('28) and since observed by others (Morelle, '31; Mason, '33; Olcott and Mattill, '34). It should be noted that his basal diet contained 15 or 20% of lard, oxidized until it was markedly rancid by aeration at 110° for 10 hours, in order to insure

[2] We are indebted to Doctors Emerson and Evans for the opportunity of reading their manuscript "The effect of vitamin E deficiency upon growth," before publication.

the absence of vitamins A and E. The former was supplied as a supplement but unfortunately the possible curative effect of a potent wheat germ oil on the paretic animals was not determined. He concluded that the origin of the paresis was a lack of vitamin E of long standing and very high degree.

In this laboratory the extent of vitamin E deficiency of the basal diet (diet E, table 1) given to the animals used for routine testing, has often been questioned. The animals are usually sleek and well nourished and show no early cessation of growth. The casein in this diet is of commercial grade; it is not extracted nor is the yeast. Lipid extracts of these failed to provide vitamin E in adequate amount for successful gestation, and young animals reared from weaning on this diet practically never demonstrate first litter fertility even though the diet is fed without any preliminary aging to produce incipient rancidity. On the other hand, cod liver oil and lard have appeared not to be invariably lacking in vitamin E (Simmonds, Becker and McCollum, '28; Nelson, Jones and Taylor, '28).

It seemed that an answer to these questions could be obtained by rearing animals on diets rigorously freed of natural fats, these being replaced by distilled mixed ethyl esters of the fatty acids from such fats, and the lipid accessories provided in the form of carotene, calciferol, and vitamin E concentrate. If female animals reared on such a diet, but without the vitamin E supplement, required no larger doses of concentrate to insure a complete gestation than those reared on the stock E-deficient diet, the equivalence of the two diets in this respect would be demonstrated. Also further information could be secured on the relation of vitamin E to growth.

EXPERIMENTAL

Rats at weaning (24 to 29 days old) were placed in individual screen bottomed cages, and given the diets outlined in table 1. The carotene, vitamin E, and cod liver oil concentrate supplements were administered by dropping the solutions directly into the animals' mouths. Ethyl linolate and wheat

TABLE 1

Diets

Diet A:	Sucrose	61.5
	Extracted casein [1]	20
	Extracted yeast [2]	10
	Salts [3]	4.5
	Ethyl esters (distilled) of hydrogenated cottonseed oil [4]	4
	Calciferol [5] 0.1 mg./kg. of diet, dissolved in ethyl esters	
	Supplements (twice weekly):	
	Carotene [5] 0.1 mg., dissolved in ethyl esters	
	Ethyl linolate 100 mg. (3 drops)[6]	

Diet B: Diet A, with a further supplement of 2 to 5 mg. of vitamin E concentrate [7] from cottonseed oil.

Diet C: Diet A, plus a vitamin E concentrate from palm oil.

Diet E: Vitamin E-deficient diet, used in routine assays (Olcott and Mattill, '34).

Sucrose	45.5	Yeast [8]	8
Lard	22	Salts	4.5
Casein [8]	18	Cod liver oil	2

Diet F: Diet A plus a concentrate of the unsaponifiable matter of cod liver oil.[9]

Diet G: Diet A plus 2 drops wheat germ oil twice a week.[10]

Diet H: Diet A except 20% lard in place of 4% ethyl esters and 16% sucrose.

Diet J: Diet A except 4% lard in place of 4% ethyl esters (no linolate supplement).

Diet K: Diet A but with 20% ethyl esters (replacing 16% sucrose).

Diet L: Diet A but with 16% of lard replacing 16% sucrose.

[1] Extracted five times with alcohol by warming on steam bath with frequent shaking. Alcohol was removed by filtering through muslin tied around the neck of the flask. The residue was extracted five times with ether and was then spread on trays and dried for 1 to 1½ hours in current of warm air.

[2] Courtesy of Northwestern Yeast Company. Extracted five times with ether as described above.

[3] Hawk and Oser ('31), Science, vol. 74, p. 369.

[4] A hydrogenated cottonseed oil was refluxed for 24 hours with twice its volume of absolute alcohol containing 2 to 3% dry hydrogen chloride. After cooling, the mixture was diluted with water and the ester layer washed repeatedly, centrifuged from occluded water, dried, and distilled in vacuo. The esters distilled below 160° at 0.1 mm. Until incorporation in the diet, they were kept cold and under carbon dioxide.

[5] We are indebted to Dr. C. E. Bills, Mead Johnson and Company, for generous supplies of these supplements.

[6] Prepared from commercial linoleic acid (Kahlbaum) as described in note 4. This was later omitted since several control feeding experiments demonstrated that the ethyl esters of cottonseed oil contained sufficient linolate.

[7] Prepared as previously described (Olcott and Mattill, '34) from cottonseed or palm oil, and fed in quantities of 2 to 5 mg. as determined by assay, such that each of the two doses per week provided enough for a fertile mating in a mature animal on the stock E-deficient diet (E).

[8] Unextracted.

[9] Four milligrams of the unsaponifiable matter of cod liver oil fed twice a week dissolved in ethyl esters.

[10] Two drops twice a week, mixed with the diet. Kindly supplied by General Mills, Inc. The assays indicated that this dosage should produce fertile animals.

germ oil were fed in a small amount of the diet, the food cups being removed until the supplement was eaten. The animals were weighed and fed the supplements twice weekly. At the end of the experimental growth period, males were sacrificed; a smear from the epididymis was examined for motile sperm and the testes were weighed and examined histologically.[3] The females were mated either before or after transfer to the stock vitamin E-deficient diet, in order to determine their fertility. Those which were deficient in vitamin E as shown by a resorption gestation, were given adequate amounts of a concentrate at the time of their second mating.

RESULTS AND DISCUSSION

The growth curves for several groups of rats are shown in figures 1 and 2. It is obvious that the animals on the highly purified diet A did not show the early cessation of growth described by Blumberg. Indeed, they grew practically as well as the colony animals whose growth curves he used for comparison. However, from the age of about 2 months the rate of growth of the male animals became progressively slower than that of the males receiving vitamin E (fig. 2). At 225 gm., the maximum weight attained by Blumberg's animals, they were still gaining about 12 gm. per week and the plateau at which growth finally ceased was at 300 gm.

Since the female animals were nearly all tested for fertility the data on growth are limited. Up to the time of the first pregnancy ($3\frac{1}{2}$ months of age), however, the rates of growth of the control animals (diet A) and of those receiving supplements of vitamin E were so similar that they have not been included in the figures. The growth curves are practically superimposable, an observation in agreement with that of Emerson and Evans ('37).[4]

Supplementing diet A with ethyl linolate (to obviate the possibility of an unsaturated fatty acid deficiency) or with a

[3] We are deeply indebted to Prof. E. Witschi of the Department of Zoölogy for his help in the preparation and interpretation of the sections.

[4] See footnote 2 on page 306.

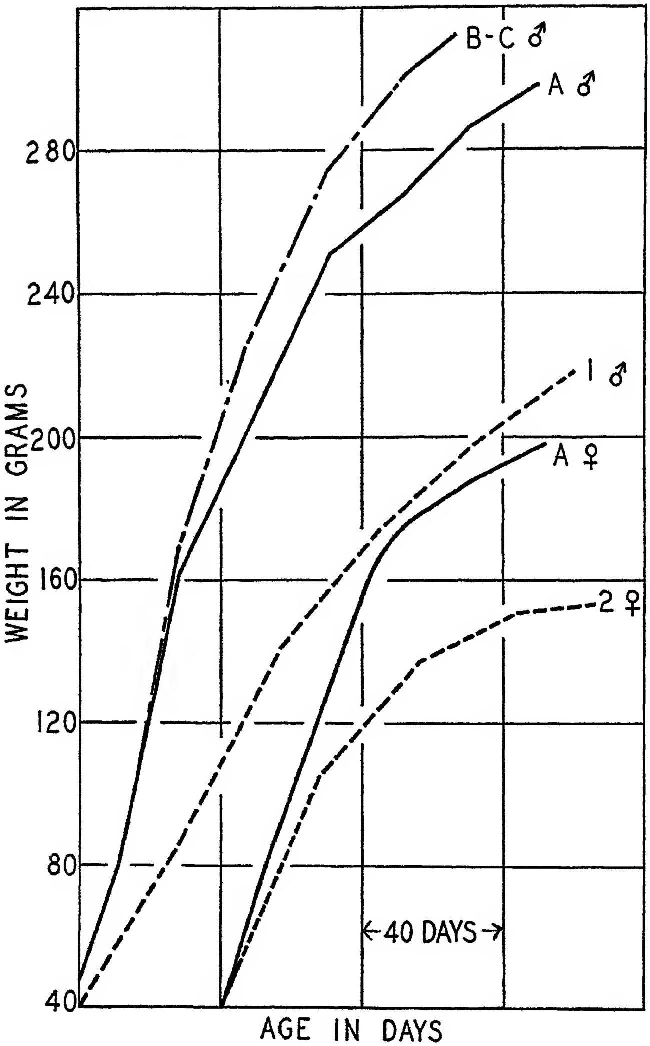

Fig. 1 Average growth curves of rats on (A) a highly purified vitamin E-free diet (twelve animals); (B–C) the same plus concentrates of vitamin E (twelve animals). In the females, vitamin E supplementation did not accelerate growth before the 110th day. (1) and (2) represent the growth curves obtained by Blumberg ('35).

cod liver oil concentrate (to augment the amounts of vitamins A and D furnished as carotene and calciferol) did not increase the growth rate or the maximum weight reached by the control animals.

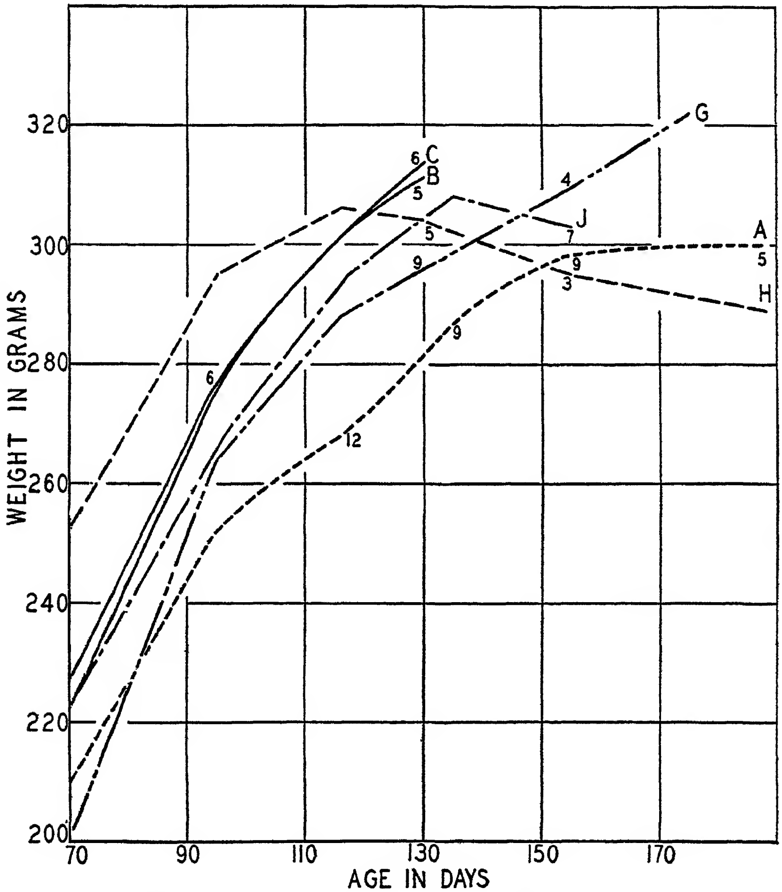

Fig. 2 Average post-adolescent growth curves of male rats on (A) the vitamin E-free ration; (B, C) the same plus vitamin E concentrate; (G) the same plus 4 drops wheat germ oil weekly. (J) contained 4% lard and (H) 20% lard instead of corresponding amounts of sucrose. The figures represent the number of animals whose weights were averaged.

On the diet (H) containing 20% lard the rate of early growth was definitely superior to that shown by rats receiving diet A, but growth ceased earlier and the maximum weights attained were comparable to those of control animals (diet A). The female animals on high lard diets, like the males, were slightly heavier during adolescent growth than the controls. The superior early growth of animals fed lard has also been noted by Burr ('36).

TABLE 2
Reproductive capacity and behavior

DIET	NO. OF ANIMALS TESTED	AGE IN DAYS	MALE ANIMALS, CONDITION OF TESTES				NO. OF ANIMALS TESTED	FEMALE ANIMALS, FIRST GESTATION		
			No. degenerated	No. normal	% weight of normal [1]	% fertility [2]		No. of resorptions	No. of births	No. of young per litter
A	12	130–303	12	0	45– 98	0.5–20	6	6	0	
B	5	130	0	5	106–127[3]		6	0	6	6– 9
C	6	130	0	6	113–145[3]		6	1	5	8–13
F	6	130	6	0	44– 54	3–10	5	5	0	
G	5	130	0	5	85–121	100	7	2	5	2– 9
G	4	175, 244	3	1?	51– 70	1–3, 75				
H	5	130–301	5	0	51–150	5–20	4	4	0	
J, K	9	166–175	9	0	48– 78	0.5–2[4]				
L	4	166	4	0	54– 74	1–33				

[1] Compared with Donaldson's tables. The occasional marked edema lessens the significance of this figure and also the correlation between this and the per cent fertility.

[2] Approximate per cent of normal tubules estimated by Doctor Witschi from his stained sections.

[3] Motile sperm; testes not sectioned.

[4] Five animals; in four animals, non-motile sperm, testes not sectioned.

A number of second generation animals, both male and female, born of mothers on E-deficient diets following the administration of adequate doses of vitamin E, were grown to sexual maturity on diets with and without supplements of the vitamin. The animals that had never had access to vitamin E grew, through adolescence, just as well as those receiving it, but thereafter, like the first generation, they lagged behind. They never developed symptoms of malnutrition or paresis.

The effects of these rations on reproductive capacity and behavior are summarized in table 2. The female animals on diets devoid of vitamin E demonstrated no first litter fertilities while, with two exceptions, those whose diets contained some vitamin E gave birth to litters at the first gestation. One of the exceptions was the lot receiving wheat germ oil supplement (diet G); the amount supplied seems to have been inadequate as appears also in the corresponding data from the male animals. One lot of these males, sacrificed at 130 days of age, was normal, while a second lot which lived longer showed extensive degeneration. The oil was not assayed again at the end of the experiment and the amount of vitamin E may have decreased through gradual deterioration of the oil. The male animals on the other diets demonstrated sterility or fertility in accordance with expectations.

The sharp contrast with respect to fertility between the animals on diet A, devoid of vitamin E, and diets B and C containing standardized amounts of a concentrate, together with the similarity of the early growth rates indicates that vitamin E is not required for adolescent growth. Whether the apparently earlier susceptibility of male than of female animals to the absence of vitamin E is a true expression of different requirements cannot yet be stated. The factors concerned in producing the disabilities described by Ringsted and Blumberg also remain to be determined; that there are other associated fat-soluble factors is evident from recent work on the vitamin B complex and on the dietary production of muscular dystrophy in herbivora. The existence of at least three specific substances having vitamin E activity [5] (Evans, Emerson and Emerson, '36) may clarify as well as complicate the situation.

It is improbable that the lard in the stock vitamin E-deficient diet is the source of any appreciable amount of vitamin E, even without undergoing preliminary rancidification. In the comparable instances, vitamin E concentrates were as effective in preventing resorptions in the animals on the diet (A)

[5] Personal communication from Dr. O. H. Emerson.

rigorously freed of all traces of vitamin E as in the animals on the stock E-deficient diet containing lard and unextracted casein and yeast. Statistical proof requires a much larger number of animals than were available but for practical and assay purposes a more rigorous purification of the E-deficient ration seems unnecessary. On the other hand, such a procedure is the only one which will yield information on the unknown fat-soluble factors of the diet which affect growth and well-being.

For the first time, apparently, a ration has been designed in which all of the lipid constituents are of known chemical composition, and on which animals can grow and reproduce normally. Lactation is, however, generally a complete failure despite the generous amounts of vitamin E administered. Such a ration should serve as a satisfactory basal diet for the study of the unknown factors in lactation.

SUMMARY

Rats were fed a synthetic diet, entirely free from vitamin E, containing extracted casein and yeast, sucrose, distilled ethyl esters of the fatty acids of hydrogenated cottonseed oil, carotene, calciferol and a salt mixture. The adolescent growth rate of the animals was approximately equal to that shown by animals receiving, in addition, highly purified concentrates of vitamin E in amounts adequate to produce fertility. This was also demonstrated by second generation animals which had never received vitamin E.

From the age of 2 months onward the male animals receiving vitamin E surpassed the controls in weight, and when they were sacrificed at 5 months of age they weighed approximately 10% more than the controls. There were no differences in the female animals at the age of 3 to 4 months.

The effectiveness of the vitamin E supplements was confirmed by the reproductive performance of the female animals and, in the case of the males, by the weight and histology of the testes.

Early growth in both sexes was accelerated by the inclusion of lard in vitamin E-deficient diets.

An uncomplicated vitamin E deficiency is not the cause of the early decline in growth rate and the appearance of serious malnutrition and paralysis recently observed by Blumberg and Ringsted; normal early growth is not dependent on the presence of vitamin E.

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THE RELATION OF ASCORBIC ACID INGESTION TO MINERAL METABOLISM IN CHILDREN

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From the data available (Holst and Frölich, '07; Bauman and Howard, '12, '17; Howard and Ingwaldsen, '17; Howe, '20, '22, '24; Robb et al., '21; Toverud, '23; Mellanby, '29; Michaux, '33), the connection between scurvy and mineral metabolism is not clear. If such a relationship exists, evidence of this should be obtained through mineral balance and vitamin C studies in man during the growth period. So far as we are aware, the only study with children in any way related to the subject is that of Chaney and Blunt ('25) in which the effects of the addition of orange juice on the mineral metabolism of two girls 10 and 11 years of age, respectively, were tested. The results suggest that the increased retentions following the orange juice ingestions may have been due to the vitamin C since the control diet was quite lacking in this essential substance and the retentions were far greater than could be accounted for by the increased mineral ingestions. On the other hand, orange juice may contain some substance or substances other than vitamin C which were instrumental in bringing about the higher retentions. Nor is it certain that the condition of the children was not an influencing factor. At the beginning of the study, the children showed "some indications of malnutrition, but were free to gain." Depleted children do not respond equally to increased food intakes. A 3-day or even a 5-day preliminary period may be insufficient

for metabolic adjustment depending upon the physiologic fullness of the tissues of the individuals under observation. The higher retentions of a second period immediately following the first may be due to a better diet in the first as well as in the second period. The data of short metabolism periods, which were used by Chaney and Blunt, may be misleading unless daily balances are made. With the exception of sulphur, Bauman and Howard ('12) obtained higher retentions during the first 2 days following the addition of orange juice to the diet of the patient suffering from scurvy than during the subsequent 5-day period, suggesting that these increases may have been due to previous depletion, the result of a poor diet immediately preceding the test, rather than the lack of the antiscorbutic vitamin per se.

EXPERIMENTAL PROCEDURE

A recent investigation of the vitamin C needs of children of preschool age (Everson and Daniels, '36) offered an opportunity to study the influence of various levels of vitamin C ingestion on mineral retentions; and although at no time were the diets of the children under observation entirely free from vitamin C, there were periods during which considerably less than the minimum requirement was being taken, as shown by the ascorbic acid retentions. The longer than usual metabolism periods consisting of 15 days each, 5 for physiologic adjustment to a given ascorbic acid ingestion level, and two successive 5-day collection periods, would seem to give significance to the findings, since this should have allowed time for the ascorbic acid to influence the mineral metabolism. Furthermore, during the twenty-one metabolism periods, covering 5 months of study, the food intake for a given child with the exception of orange juice or commercial ascorbic acid when used, was very constant, and insofar as known, adequate. The same amounts of the various foods for a given child were used in all periods, excepting in those cases when slight increases were made to meet the caloric needs of the children. Conditions affected by variations in vitamin D would seem to

have been ruled out by the daily ingestions of cod liver oil and viosterol, supplying approximately 3000 units (U.S.P.), the 10-minute irradiation with a mercury quartz lamp, and a playroom equipped with corex glass windows. Comparisons of mineral retentions during the various metabolism periods with the time spent out of doors indicated that this was without influence in this particular study.

The details of the study, including methods of testing the ascorbic acid intake and excretion, the preparation of the food and general care of the children are given in the former paper (Everson and Daniels). Nitrogen, phosphorus, calcium and magnesium determinations with the exception of the calcium and magnesium of the urine, were made by the methods in a previous report (Daniels et al., '35). The work was started with the idea that the urinary calcium excretions at the same levels of ingestion would be fairly comparable for a given child. The variations during the first periods led to a study of the calcium of the urines by the more frequently used method of McCrudden ('09) and that of Washburn and Shear ('32) with ashed urine. Checks of repeated tests with the latter method were found to be more nearly uniform, although in many instances results by the two methods were the same. After somewhat extensive tests, the method of Washburn and Shear became the method of choice.

Nitrogen determinations, in each case, were made with the 5-day pooled urines. The addition of 2 to 5 drops of sulphuric acid to each urine specimen for the purpose of preserving the ascorbic acid resulted in more or less acid urines; therefore, each day after these were made up to volume, enough acid was added to bring the total to a pH of 2.7 to 3. On standing, the uric acid precipitated. This was filtered off before aliquots were taken for calcium and nitrogen determinations. Thus the urinary nitrogens are slightly less than their true values and, the nitrogen retentions correspondingly higher; but since the same technics were used in all cases, the slight error introduced is the same in each balance reported.

For further study of the influence of vitamin C on mineral metabolism, we have assembled data from previous balance studies in which the ascorbic acid was obtained chiefly from weighed amounts of orange juice and canned tomatoes. The vitamin C content of these and of the other foods which contained minimal amounts was not determined directly. The figures used are estimates based on the averages of the determinations in the balance study, or as with tomatoes, a subsequent determination. We appreciate this introduces some error, but not enough, we believe, to invalidate the results, especially since the majority of the ascorbic acid ingestions were found to be considerably in excess of the minimum requirement.

These selected studies have differed from those of the ascorbic acid balance study in that different amounts of calcium, from 1 quart or 1 pint of milk, respectively, were taken as well as different amounts of orange juice. It seemed possible that the influence of ascorbic acid on calcium retentions might be more obvious at lower levels of calcium ingestion. Similarly, the amount of orange juice as such, 60 cc. and 120 cc., may have influenced the retentions. The children studied were of the same age, and during the metabolism periods lived under the same controlled conditions for the same length of time as did those of the ascorbic acid balance study. Cod liver oil and viosterol also were given daily, the same amounts and brands being used in all cases. The orange juice was divided into two parts, one portion being given at 10 A.M., and the other between 3.30 and 4 P.M.

RESULTS

The findings of the ascorbic acid balance study (Everson and Daniels) indicate that under the conditions of the study optimum retentions of ascorbic acid are obtained in children of the age considered (39 to 59 months) when from 6 to 7.5 mg. per kilogram are included in the diet. Ingestions of greater magnitude resulted in no higher retentions. If mineral retentions, more particularly calcium, phosphorus and magnesium are influenced by ascorbic acid ingestions, it would

seem that low values should be obtained when the intakes fall below 6 mg., and consistently high retentions with ingestions above 7.5 mg. at a given calcium ingestion level.

The mineral retentions of the children receiving the constant diets which varied only in the amounts of orange juice and vitamin C suggest that there is no direct relation between ascorbic acid retention and calcium, phosphorus and magnesium retention within the limits of the study. With the exception of nitrogen the average retentions with ascorbic acid ingestions below 4.5 mg. per kilogram were the same as those during ingestions of 6.7 to 11.5 mg. per kilogram (table 1). This lack of relationship between ascorbic acid at the levels tested and mineral metabolism is further indicated by the results with both the higher and lower levels of mineral, more particularly calcium, ingestion (table 2). The highest calcium value obtained, 17.5 mg. per kilogram occurred during an ascorbic acid intake of 7.2 mg. per kilogram with 80 mg. of calcium per kilogram, whereas 13.5 mg. were retained during an ingestion of 8.3 mg. of ascorbic acid and 51 mg. of calcium. Ascorbic acid ingestions of twice the physiologic minimum resulted in no higher calcium retentions than when just enough to cover the minimum requirement was being taken.

That orange juice as such in the amounts tested has no influence on mineral retentions when included in a diet which contains insofar as is known adequate amounts of all the known essential constituents is shown both by the ascorbic acid balance studies and the controlled ascorbic acid studies. The minimum orange juice tested was 30 cc., the optimum 120 cc. At these two levels of ingestion, calcium and phosphorus retentions were practically the same (table 1) nor were the retentions in the controlled balance study significantly different at the various levels of ingestion (table 2).

The most suggestive findings of the ascorbic acid balance study are concerned with the nitrogen metabolism. The average retention during the period of low intake is 27.1% less than that of the higher level (table 1). These lower retentions of the first group are the more significant since of these, three

TABLE 1

Influence of ascorbic acid ingestion on the mineral metabolism of children receiving constant diets

NUMBER OF PERIODS	AVERAGE WEIGHT	INGESTION							RETENTION PER KILOGRAM										AVERAGE OUTDOOR PLAY PER DAY
				Average per kilogram					Ascorbic acid		Calcium		Phosphorus		Nitrogen		Magnesium		
		Orange juice	Redoxon [1]	Ascorbic acid	Calcium	Phosphorus	Nitrogen	Magnesium	Average	Range	Average	Range	Average	Range	Average	Range	Average	Range	
	kg.	*cc.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*min.*
3[2]	18.2	30	..	2.8	64	67	513	13.5	2.1	2.0–2.4	9.2	6.9–10.6	5.2	3.9–5.9	36	21–43	0.6	0.4–0.8	21
3[3]	18.7	60	..	3.7	63	71	553	14.6	2.3	1.9–2.6	10.0	8.7–11.3	6.2	4.7–8.6	41	20–62	1.1	0.6–1.4	61
3[3]	17.9	60	..	3.9	66	72	556	14.7	2.7	2.6–2.7	9.1	7.0–10.8	6.3	5.4–7.2	55	53–58	0.9	0.7–1.0	60
3[3]	15.6	60	..	4.4	72	75	550	15.3	3.0	2.8–3.2	11.2	7.9–14.7	6.3	5.5–7.1	40	33–46	1.0	0.7–1.3	60
Average				*3.7*	*66*	*71*	*543*	*14.5*	*2.5*		*9.9*		*6.0*		*43*		*0.9*		*51*
3[2]	16.9	60	48	6.7	68	74	560	14.8	3.3	3.0–3.7	11.2	10.3–12.4	5.4	4.5–6.1	56	42–64	0.5	0 –1.0	80
3[2]	17.4	120	..	6.9	67	73	550	15.3	3.9	3.6–4.2	8.9	6.9–10.2	5.7	5.4–6.2	55	49–59	1.1	0.9–1.3	36
3[2]	18.3	120	96	11.5	65	69	530	14.3	3.5	3.2–4.0	10.3	6.2–15.5	6.2	5.8–6.7	65	51–86	1.1	0.9–1.4	0
Average				*8.4*	*67*	*72*	*546*	*14.8*	*3.6*		*10.1*		*5.8*		*59*		*0.9*		*39*

[1] Commercial ascorbic acid—Hoffman-La Roche Co.

[2] Averages are the results obtained with the three children studied.

[3] In each case, the averages are the results obtained with a given child.

TABLE 2

Relation of ascorbic acid ingestion to mineral retentions of children receiving diets containing two levels of milk

NUMBER OF CASES	AVERAGE WEIGHT	INGESTION PER KILOGRAM				RETENTION PER KILOGRAM					
		Ascorbic acid, average	Calcium, average	Phosphorus, average	Nitrogen, average	Calcium		Phosphorus		Nitrogen	
						Average	Range	Average	Range	Average	Range
Diets containing 486 gm. milk											
	kg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1	14.3	12.2[1]	54	76	570	8.5	....	6.2	....	80	......
4	15.6	11.4[1]	53	69	562	10.2	6.5–12.4	7.7	4.5–10.0	90	56–120
2	16.2	10.4[1]	51	65	550	10.1	9.9–10.4	5.9	4.0– 7.9	78	78– 78
2	14.4	9.2[2]	56	64	510	6.1	5.8– 6.5	6.0	5.6– 6.4	83	57–110
5	16.0	8.3[2]	51	63	490	10.3	6.9–13.5	9.3	7.7–11.3	86	77– 96
5	17.1	7.2[2]	48	61	478	8.2	6.0–10.1	5.4	2.9– 6.7	60	38– 77
3	19.6	6.4[2]	44	56	450	8.6	7.4– 9.9	4.6	3.3– 5.8	41	39– 47
1	19.9	5.9[2]	42	56	470	6.5	....	1.8	....	41	......
Diets containing 976 gm. milk											
1	14.7	12.3[1]	90	90	580	14.8	....	5.0	....	67	......
2	15.8	11.3[1]	80	81	550	10.3	9.9–10.7	6.1	4.5– 7.7	84	76– 92
1	16.6	10.6[1]	81	78	540	11.2	....	8.5	....	98	......
1	14.0	9.1[2]	98	84	570	11.1	....	15.7	....	75	......
3	14.8	8.6[2]	88	83	570	12.1	9.4–14.8	6.8	4.4–10.0	68	50– 91
4	16.9	7.2[2]	80	78	570	10.6	7.8–17.5	6.5	4.9– 8.3	60	50– 76
4	19.6	6.5[2]	72	75	555	8.8	6.9–10.3	4.9	3.5– 7.6	50	46– 54

[1] Diets included 120 cc. orange juice.
[2] Diets included 60 cc. orange juice.

balance studies were the first metabolism periods of the series and three other periods immediately following the 3-week Christmas holiday—periods when it is not unusual to obtain high retentions which we have attributed to the less well controlled home diets of the children previous to entrance to the metabolism ward. Because of past experience, neither the highest nor lowest ascorbic acid ingestions were tested at these times. Nitrogen retentions in the balance studies in which the ascorbic acid ingestions were estimated (table 2) indicate that with few exceptions sufficient ascorbic acid had been taken. The average nitrogen retentions are not consistently related to ascorbic acid ingestion. The range of retentions, furthermore, shows that high retentions may be obtained at any level of ingestion above 6.4 mg. per kilogram, or thereabout. The low retentions at the lowest ingestion levels are in line with the findings of the ascorbic acid balance study.

COMMENTS

In evaluating the effect of orange juice on mineral retentions, it should be noted that in no case was all of the ascorbic acid obtained from orange juice. Given amounts of raw banana were included in all diets and with the exception of the ascorbic acid balance study, all diets contained some canned tomatoes. In diets containing all the known essential substances in seemingly adequate amounts, orange juice apparently is without influence on the calcium, phosphorus and magnesium retention. With other types of diets, orange juice may contribute some substance or substances essential to mineral metabolism, which in our diets were obtained from other foods. Without more knowledge, one hesitates to hazard a conjecture as to what substance beside vitamin C was below the physiological minimum in the Chaney and Blunt diets. Their basal diets were neutral, changing to basic with the addition of orange juice, whereas those used for the ascorbic acid studies were basic at all levels of orange juice ingestion. Vitamin D, in the Chaney and Blunt study was uncontrolled and not given as such, and although "the children led a regular

happy life with plenty of out-door sunshine,'' more out-of-doors may have been available during the orange juice periods, thus accounting for the higher retentions during that period; or, as has been suggested, the increased retentions may have been due to a stimulation of growth resulting from the better diets of both periods, the effects being more evident in the second. There seems to be no evidence either in the studies herein reported, or in those of Bauman and Howard to support the thesis that in man vitamin C is directly concerned with calcium and magnesium metabolism. Indirectly, when not present in sufficient amounts over considerable periods, it may be related by decreasing the oxidative capacity of the tissues (Harrison, '33) and by bringing about such profound tissue changes (Howe, '20; Wolbach and Howe, '26; Menkin, Wolbach and Menkin, '33; Siehrs and Miller, '34; Park et al., '35) that these essential mineral substances cannot be fixed.

In line with these tissue changes are the findings of Bauman and Howard ('12), Howard and Ingwaldsen ('17), and Kachevnik and Eidman ('35), suggesting that scurvy is a disease primarily affecting nitrogen metabolism. With the patient suffering from scurvy, nitrogen retentions which were negative when the patient was receiving a diet containing no vitamin C became positive when orange juice was added to the diet. With the monkey and the guinea pigs in which scurvy was developed, the nitrogen excretions increased as the disease progressed. Chaney and Blunt found higher nitrogen retentions in the children studied when orange juice was added to the diet. The lower nitrogen retentions of the children receiving less than the physiological minimum of vitamin C in the ascorbic acid balance study corroborate these findings and offer an explanation for those studies in which growth was stimulated by the addition of orange juice to the diets of underweight children (Chaney, '23; Morgan et al., '26).

We began the study anticipating that at low vitamin C levels, calcium metabolism might be affected, therefore the results were the more surprising, as were the findings of the influence on nitrogen retentions of vitamin C at ingestion levels

which we have hitherto believed to be adequate. It is not customary for children to be given as much vitamin C as the ascorbic acid balance study indicates they should have, one raw fruit a day being the usual dietary dictum. One is led to consider what proportion of children are suffering from undernutrition because of too little vitamin C of the diet and what the ultimate effects of less than the physiological requirement over a considerable period will be. Studies with guinea pigs have seemed to point to a relation between vitamin C, calcium metabolism and dental caries. In man, this relationship has not been established. In view of our results, it seems possible that a continued ingestion of less than the optimum amount of vitamin C may so affect the dental tissues that calcium will fail to be retained. Further work is needed to confirm this hypothesis.

SUMMARY

Calcium, phosphorus, magnesium and nitrogen retentions in children of preschool age have been studied in relation to ascorbic acid ingestions and retentions during periods when the children were receiving diets which included 1 quart, 3 cups and 1 pint of milk, respectively. The influence of two levels of orange juice ingestion, 60 cc. and 120 cc., also has been considered. Variation in vitamin D as an influencing factor was seemingly ruled out since each child received approximately 3000 U. S. P. units per day. Each child, furthermore, was given a 10-minute exposure to ultra violet light from a mercury quartz lamp and lived in a room equipped with corex glass windows. The time spent out of doors showed no correlation with the retentions.

The findings indicate that within the limits of the study:

1. Ascorbic acid ingestions between 2.7 and 12.5 mg. per kilogram have no influence on calcium, phosphorus and magnesium retentions. In the controlled ascorbic acid balance study in which the ascorbic acid retentions were shown to be below the physiologic minimum, there was found to be no appreciable difference in the average retentions of calcium, phosphorus

and magnesium. Individual differences noted were not related to the ascorbic acid ingestions.

2. There is no apparent relationship between the calcium and phosphorus retentions and the calcium and ascorbic acid of the diet when more than the physiological minimum of either is taken.

3. Ascorbic acid seems to be related to nitrogen metabolism. The average nitrogen retentions at levels of ascorbic acid ingestions below the physiological minimum were found to be lower than when the children were receiving adequate amounts as shown by the ascorbic acid retentions. Ascorbic acid ingestions in excess of the physiologic requirement, however, do not further increase the nitrogen retention.

4. Orange juice per se, at the levels tested, 60 cc. and 120 cc. per day, when given in conjunction with diets containing insofar as is known all the essential constituents in adequate amounts, is without influence on the calcium, phosphorus, magnesium and nitrogen retentions.

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THE EFFECT OF THE QUALITY OF PROTEIN ON THE ESTROUS CYCLE [1]

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SIX FIGURES

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Aberrations of the estrous cycle due to nutritional deficiencies have been reported by various workers. Fraenkel ('17) reported that an insufficient food supply may lead to amenorrhea and sterility in humans. Evans and Bishop ('22), Marrian and Parkes ('30), and Asdell and Crowell ('35) showed that partial inanition interfered with the normal estrous phenomena in the rat. Working with guinea pigs Loeb ('17) found that underfeeding caused a disturbance of the estrous cycle and a hypotypical condition of the ovaries. This hypotypical condition was characterized by a failure of the follicles to mature and by atresia occurring before the follicles reached medium size.

Only a limited amount of information on the effect of the quality of proteins on the sexual behavior of the animal is available. Courrier and Raynaud ('32) found that when rats were fed a diet containing gliadin as the main source of protein the animals failed to ovulate but remained in anestrus. Estrus was induced by implantation of an hypophysis of a normal rat into an animal that was in anestrus. On the basis of this evidence these workers concluded that lysine was necessary for ovulation. Osborne and Mendel ('12) reported

[1] Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

that feeding a diet deficient in lysine does not impair the capacity of the animal for reproduction. Their evidence was, however, based on the performance of a single animal. Recently Pearson ('37) has further investigated the influence of a lysine-deficient diet containing 18% of gliadin, which was the sole source of protein except for the small amount that may have been present in the vitamin addenda. Except for the protein the diet contained all essential ingredients necessary for growth. Rats that were fed this diet remained in anestrus; when a preparation of hexone bases or *d*-lysine dihydrochloride was added to the diet normal estrous cycles were resumed. There was no evidence from this work that lysine possessed a specific stimulatory function in maintaining the normal state of the estrous cycle.

In order to throw further light on the effect of the quality of proteins on the estrous cycle and to deduce information as to whether or not a deficiency of other amino acids may not cause an aberration similar to a lysine deficiency, further investigations along this line were undertaken. The estrous behavior has also been correlated with the histologic picture of the ovary.

EXPERIMENTAL

The diets employed are presented in table 1. Gliadin was prepared by extracting wheat gluten with 70% ethyl alcohol. Reprecipitated casein was used. In order to furnish ample amounts of the B factors a 25% acidulated alcoholic extract of brewers' yeast was evaporated on the dextrin at a level equivalent to 2% of yeast. One-half milliliter of liver extract was fed individually each day. The liver extract was prepared from an anhydrous liver concentrate manufactured by Wilson and Company. The powdered liver was dissolved in 2 volumes of water. Five volumes of ethyl ether and 6 volumes of ethyl alcohol were then added and allowed to stand for several hours with frequent shaking. The solvent was then decanted and the precipitate dissolved in water and made up to a volume so that 4 ml. were equivalent to 1 gm. of the liver powder. This material was allowed to stand for

several hours and then centrifuged. The solution was again made up to volume so that 4 ml. were equivalent to 1 gm. of the original liver powder.

The animals used were fed a stock diet until they reached sexual maturity and presented normal estrous cycles. The stage of the estrous cycle was followed by means of daily microscopic examination of the vaginal smear (Long and Evans, '22). The animals in two of the groups were not changed to the restricted diet until they were 100 days or more of age, while a third group was changed at the second period of estrus, which occurred at approximately 50 days

TABLE 1

Percentage composition of diets

	DIET NO.			
	101-P	102-P	104-P	105-P
Dextrin	89.0	84.0	79.0	84.0
Casein	5.0	5.0	5.0	5.0
Gliadin	..	..	..	5.0
Gelatin	..	5.0	10.0	..
Salts [1]	4.0	4.0	4.0	4.0
Cod liver oil	1.0	1.0	1.0	1.0
Wheat germ oil	1.0	1.0	1.0	1.0
Per cent protein	4.82	9.72	14.62	9.70

[1] Phillips and Hart ('35).

of age. This afforded an opportunity to observe the effect of restricted protein intake on the estrous behavior of animals that were practically mature as compared with animals that were immature in respect to growth. Groups of animals were fed ad libitum on a diet containing 5% of casein until the effect on the estrous cycle had been definitely established. Gelatin or gliadin was then added to the diet and the response of the animals observed.

Supplementary effect of gelatin. A group of six rats 105 days of age was placed on diet 101-P containing 5% of casein. The estrous behavior and the average weight of the animals are presented graphically in figure 1. The weight decrement

starting about the fourth week is believed to be due to the use of a liver extract that was impotent. As the animals regained their weight, when a new preparation of liver extract was fed, they were continued in the experiment. There is no reason to believe that this divergence in weight affected the subsequent behavior of the animals in respct to the estrous cycle when gelatin was added to the diet.

It is recognized that a deficiency of the vitamin B factors may have accentuated the aberrations observed during this

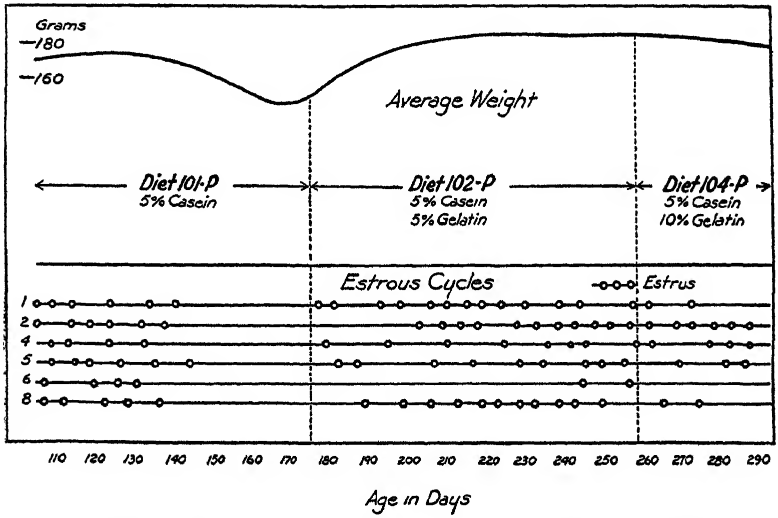

Fig. 1 Weight curve and estrous behavior of rats on diets quantitatively and qualitatively inadequate in protein. The period of estrus is indicated by a circle.

period. In order to clarify this point another group of rats was fed for a period of 10 weeks on diet 101-P. These animals were started on the experimental diet at 110 days of age. Their average weight at the beginning was 190 gm. and after 10 weeks on the low protein diet they weighed an average of 188 gm. All of the animals showed marked aberrations of the estrous cycle after 30 days. In some cases there was complete cessation of estrous cycles, while in others estrus occurred at prolonged and irregular intervals.

Since casein is low in the sulfur bearing amino acids, it might be expected that an addition of cystine to the diet would restore the estrous cycle. However, it has been shown (Pearson, '36) that adding cystine to such a diet was without effect with respect to either the estrous behavior or weight. As it has been demonstrated that lysine is essential for maintaining the normal sexual rhythm we decided to add 5% of gelatin, which is an incomplete protein but relatively high in lysine. According to the analyses of gelatin (Foster and Schmidt, '23; Knoop and Oesterlin, '25) this amount should supply adequate lysine for normal functioning of the animals. After 5% of gelatin was added to the diet none of the animals except no. 2 presented normal estrous cycles. Increasing the gelatin to 10% was without further beneficial effect as there was no significant change in the sexual behavior of the animals after they were transferred to 104-P. At best, the addition of gelatin brings about only a partial response in the estrous cycle. Assuming that the gelatin furnishes ample lysine, there is no evidence that lysine plays a stimulatory role in the sexual phenomena. It does appear, however, that one or more other essential amino acids inhibit normal sexual function. Further evidence to support this postulation is deduced by supplementing a low casein diet with gliadin.

Supplementary effect of gliadin. The animals used in this trial were fed a standard stock diet until the occurrence of the second estrus, at which time they were changed to diet 101-P containing 5% casein. These animals weighed on the average approximately 155 gm. and were considerably younger when placed on the experimental diet than were the animals in the previous group. The performance of these animals is summarized in figure 2 by means of a composite growth curve and a graphical presentation of the estrous cycles. The animals presented one or two normal estrous cycles after being placed on the experimental diet. Subsequently, the periods of anestrus were much longer than normal and in some cases there was complete cessation of the estrous cycle. The animals were continued on diet 101-P for

approximately 70 days, at which time they were changed to diet 105-P which differed from the former diet in that 5% of gliadin was added in place of an equivalent amount of dextrin. It was early demonstrated by Osborne and Mendel ('12, '16) that gliadin is deficient in lysine. On the basis of our knowledge of the amino acid content of gliadin it may be concluded that diet 105-P was not materially higher in lysine than was diet 101-P, but that the content of other essential amino acids was materially increased.

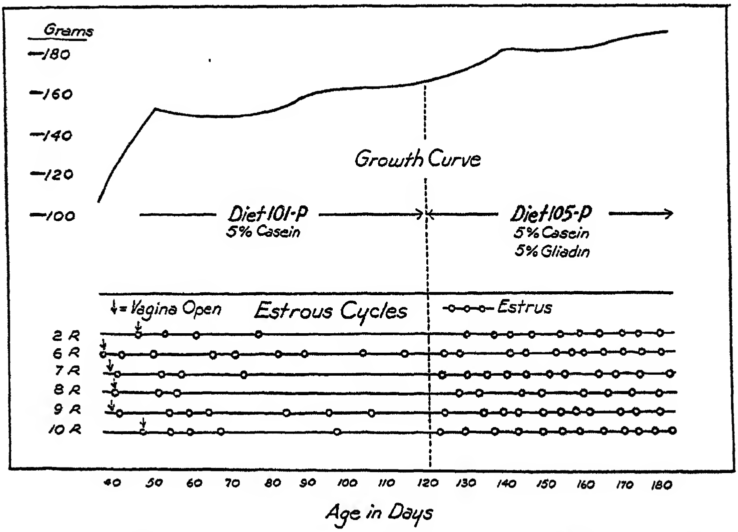

Fig. 2 The effect of adding 5% of gliadin to the diet of rats that have exhibited aberrations of the estrous cycle after being restricted to a diet containing 5% of casein as the sole source of protein. Estrus in indicated by a circle.

From figure 2 it is seen that with the addition of 5% of gliadin to the diet all of the animals passed from anestrus to estrus and continued, thereafter, to present normal estrous cycles. Concomitantly with the resumption of normal estrous behavior there was an increase in weight. The animals were continued on diet 105-P for approximately 62 days. At this time the experiment was concluded and the ovaries were removed for histologic study.

Since there was only a partial response when gelatin, rich in lysine, was added to the diet but when gliadin, low in lysine, was added normal estrous cycles were immediately resumed, it seems apparent that other amino acids are equally as important as lysine in maintaining the normal sexual rhythm, and that a lack of other essential amino acids has a similar effect in causing an aberration of the estrous cycle. We recognize the fact that further work might be done on the effect of the quality of proteins in relation to the estrous phenomena. However, present knowledge appears to warrant the hypothesis that when the dietary protein is inadequate for growth there occurs a disturbance of the estrous cycle and reproductive functions.

Histologic studies. Ovaries from several animals in anestrus and others that had resumed normal cycles after being transferred to diet 105-P were studied histologically in order to correlate the vaginal smear picture with the activity of the ovary, and to observe the changes caused by diets in which the protein is either quantitatively or qualitatively inadequate. The ovaries were fixed in Bouin's fluid, sectioned serially, and stained in hematoxylin and eosin.

The whole reproductive system of the animals that had failed to come into estrus was found to be in a resting or atrophic condition. There were comparatively few primordial follicles; these, together with many of the medium-sized follicles showed various stages of atresia similar to that described by Marrian and Parkes ('30) in partial inanition and vitamin B deficiency, and by Guilbert and Goss ('32) on low protein diets. The most striking feature of the hypotypical ovaries (figs. 3 and 4) was the absence of new corpora lutea. The small shrunken bodies were obviously in the process of involution. The large follicles frequently showed vacuolar degeneration and sloughing off of pycnotic granulosa cells into the antrum. It was evident that ovulation had not taken place during the period of anestrus as recorded by the vaginal smear. In one case the left ovary was removed from a rat that had been on a diet containing 5% of casein and

had been in anestrus 42 days. This was a hypotypical ovary (fig. 4) of the first order with a large amount of interstitial tissue and no large follicles or corpora lutea. The animal came into estrus, as diagnosed by the vaginal smear, 22 days after the laparotomy and then passed into anestrus.

The ovaries of the animals in figure 2, which had showed marked aberration of the estrous cycle when restricted to the 5% casein diet, but which immediately resumed normal estrous cycles when transferred to diet 105-P, appeared normal and active. Large numbers of new corpora lutea were present (fig. 5) and there were many follicles in various stages of development. There was no doubt that ovulation had occurred in a normal manner from these previously degenerate ovaries. In fact, in one animal, 6R, figure 6, an ovum was found in the fallopian tube. From this remarkable recovery of these once degenerate ovaries it is apparent that permanent sterility is not caused by protein deficient regimens, and the follicles are capable of growth and ovulation when the required nutritive stimulus is provided.

From a study of available literature it is apparent that the hypotypical condition of the ovary is similar in all nutritionally inadequate regimens causing cessation of the estrous cycle.

Fig. 3 Photomicrograph of section of ovary of rat no. 8 which had been in anestrus 18 days. The atretic follicles and the absence of new corpora lutea are characteristic of the failure of ovulation due to inadequate nutrition.

Fig. 4 Photomicrograph of section of ovary of rat restricted to diet containing 5% of casein as the main source of protein. This animal had been in anestrus 42 days; the advanced stage of degeneration is characterized by the absence of developing follicles, corpora lutea and excess interstitial tissue.

Fig. 5 Photomicrograph of section of ovary of 6R which had exhibited marked aberration of the estrous cycle on diet 101-P, and then resumed normal cycles when 5% of gliadin was added. The young corpora lutea and normal developing follicles denote ovarian activity and ovulation.

Fig. 6 Photomicrograph of section of oviduct of 6R showing recently extruded ovum.


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SUMMARY

Rats maintained on a diet containing 5% of casein as the chief source of protein soon cease to exhibit the characteristic vaginal changes of estrus. The anestrous period is correlated with hypotypical ovaries and atrophic condition of the whole reproductive system. The failure of ovulation is indicated by atretic follicles and the absence of new corpora lutea.

The addition of gelatin, rich in lysine, brings about only a partial response in the estrous cycle. When 5% of gliadin, poor in lysine, is added to the low casein diet ovulation occurs and the normal sexual rhythm is immediately resumed. It is evident that a deficiency of amino acids other than lysine causes an aberration of the estrous cycle, and it seems that when the dietary protein is inadequate for growth the sexual behavior is likewise adversely influenced. No permanent sterility results from feeding diets qualitatively or quantitatively deficient in protein if the necessary stimulus for ovulation is subsequently provided.

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STUDIES ON THE ENERGY METABOLISM OF THE HEN [1]

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FOUR FIGURES

(Received for publication April 7, 1937)

Work on the gaseous exchange of the chicken was begun several years ago by the writer in response to the need, by a group of workers collaborating on a poultry ventilation project, for data on oxygen consumption and carbon dioxide production. Soon after work on the gaseous exchange was initiated, it seemed wise to broaden the studies to include other phases of the energy metabolism of chickens. This paper is concerned chiefly with: the effect of fasting on the energy metabolism, the basal metabolism, the heat loss due to vaporization of water, the relationship between insensible loss and metabolic rate, and the effect of egg production on the metabolic rate.

Since several recent reports on the energy metabolism of the hen (Mitchell and Haines, '27 a, b; Mitchell, Card and Haines, '27; Benedict, Landauer and Fox, '32) contain numerous references to the literature, it is not considered necessary to treat the literature extensively in the present paper. Only those papers having a direct bearing on the problems at hand will be mentioned.

[1] Presented in preliminary form at the Washington meeting of the American Physiological Society, March, 1936.

[2] Now at Cornell University.

METHOD

The Haldane (1892) gravimetric method of measuring the respiratory exchange was used. It was customary to have the water absorbers in pairs. In this way the second bottle could be used to check the efficiency of absorption by the first. The efficiency of the carbon dioxide absorption was checked either by having the absorber bottles in duplicate or by causing the air from the bottles to pass through a solution of barium hydroxide. Blank experiments (twenty-eight in number) indicated that the average error in the oxygen determination was not greater than 2%. Carpenter ('28) has shown by means of alcohol check tests that the Haldane method will give reliable results with adult poultry if the periods are sufficiently long—at least 2 hours. Most of the periods in the present work were 2 hours long.

The preliminary periods were usually ½ hour in duration. The rate of ventilation was kept constant throughout an experiment at 3 liters per minute. This is sufficient to prevent condensation of moisture in the chamber.

The heat production was calculated in the usual way. Determinations of the nitrogen metabolism were not made. Where the respiratory quotient was greater than 1, the carbon dioxide in excess of the oxygen was given a heat value of 1.09 calories per liter (Lusk, '28). Only five tests showed respiratory quotients above unity. Where the respiratory quotient was less than 0.707 but not less than 0.68, a respiratory quotient of 0.707 was assumed in making the calculations. The number of tests in this group was twenty-eight. Where the respiratory quotient was less than 0.68, the test was discarded. Only three tests were rejected for this reason.

The surface area of the birds was estimated according to the well-known Meeh formula ($S = k \times W^{2/3}$), 0.10 being used for the value of k (Benedict, Landauer and Fox, '32).

Activity records were made in most of the tests. The animal chamber was so suspended that when the bird was active the chamber would move up and down at one end. These movements were picked up by a receiving tambour and trans-

mitted to a recording tambour arranged to write on a kymograph. The activity records were divided into three classes depending upon whether the hens were quiet (I), moderately active (II) or active (III). Upon examination of the data it was evident that when the activity had been estimated to be II the metabolic rate was so little increased over the rate when the activity had been estimated to be I that the results could be placed in the same group from the standpoint of activity. Results obtained during periods in which the activity had been judged to be III were rejected (except in the three instances identifiable in table 1). Twenty-two tests were discarded because of an activity of III.

The environmental temperatures in the different experiments ranged from 21 to 29°C. The lowest of these figures is well above the critical temperature of the hen as determined by Mitchell and Haines ('27 a). Most of the experiments came within the temperature range of 23 to 27°C. During a given experiment the temperature range was usually much narrower than this.

Early in the course of this work the practice was adopted of standardizing the amount of food in the crop at the beginning of a fast. This was accomplished by removing the hens from the hen house overnight and the next morning (at which time the crop was usually found to be empty) feeding them 40 gm. of corn. The time elapsing between the feeding of the corn and the middle of the test was considered to be the duration of the fast.

The weighings were done on a 10 kg. Sauter balance having a sensitivity of 10 to 20 mg. depending on the load.

One hundred and fifty-six trials were run. Fifty-four hens (mostly Plymouth Rocks) were used. Only the results in day experiments are reported.

RESULTS

The effect of fasting on the respiratory quotient and the metabolic rate. This question was studied in fifty tests on eight Plymouth Rock hens 10 to 13 months old. The results

are shown in figure 1. It is evident that the average respiratory quotient has fallen to 0.74 by the end of the first day and to 0.70 by the end of the second day. From that time on, it remains practically constant. The average metabolic rate,

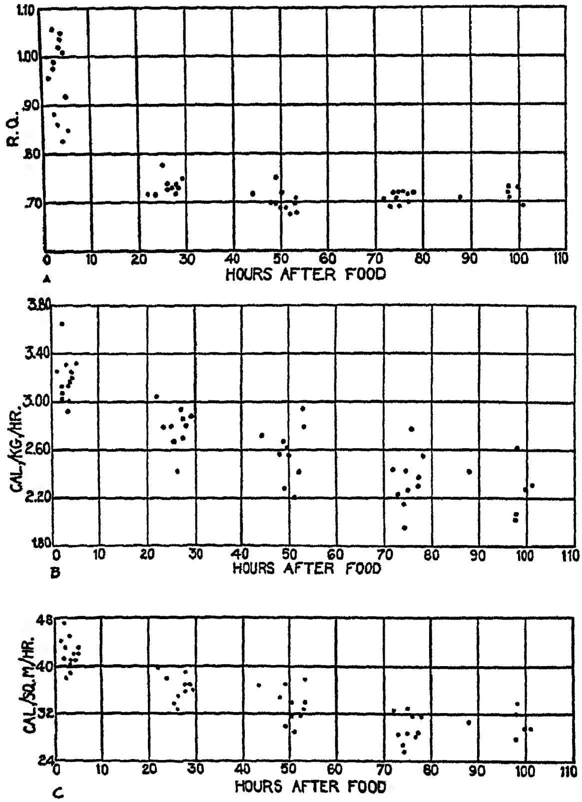

Fig. 1 The effect of fasting on the respiratory quotient and the metabolic rate. Fifty tests on eight Plymouth Rock hens, 10 to 13 months old.

both on a body weight basis and on a surface area basis, shows a progressive fall until about the seventy-fifth hour. From then on, the rate appears to be practically constant.

The basal metabolism. To obtain further data on the respiratory quotient and the metabolic rate after a fast of approximately 24 hours, fifty-four tests were run on thirty-two White

Plymouth Rock hens ranging in age from 18 to 22 months and in weight from 1.95 to 3.28 kg. (average weight, 2.51±0.03 kg.). The fasts were 23 to 32 hours in duration. The results are shown in the accompanying histograms (fig. 2). It is evident that with several distinct exceptions the data are distributed close to the normal.

TABLE 1

The R.Q. and metabolic rate 23 to 29 hours after food
(Plymouth Rock and White Wyandotte hens)

HEN NO.	AGE (MONTHS)	HOURS WITHOUT FOOD	R.Q.	CAL./KG./HR.	CAL./SQ.M./HR. ($s = 0.10 \times W^{2/3}$)	ACTIVITY	HEN NO.	AGE (MONTHS)	HOURS WITHOUT FOOD	R.Q.	CAL./KG./HR.	CAL./SQ.M./HR. ($s = 0.10 \times W^{2/3}$)	ACTIVITY
67	12	25	0.71	2.76	35.51	I	1662	12	23	0.72	2.87	35.88	I
		27	0.72	2.68	34.53	I			25	0.77	2.85	35.62	I
		29	0.70	2.66	34.14	I							
57	12	25	0.75	2.91	34.06	?	44	14	24	0.74	2.27	30.97	I
		27	0.78	2.73	31.92	I			26	0.71	2.22	30.32	I
		29	0.75	2.72	31.70	I			28	0.71	2.23	30.51	I
697	Old	25	0.74	2.18	28.22	II	2430	22	26	0.69	2.36	32.93	II
		27	0.75	2.34	30.12	II			29	0.68	2.15	30.15	I
		29	0.76	2.30	29.62	II							
30	12	24	0.73	2.29	31.15	I	2440	22	24	0.75	2.45	35.94	II
		26	0.71	2.26	30.67	I			26	0.70	2.28	33.25	I
		28	0.73	2.40	32.62	III			28	0.70	2.18	31.93	I
27	12	25	0.73	2.27	29.67	I	1562	22	25	0.79	2.85	38.23	I
		27	0.73	2.33	30.48	I			27	0.77	3.32	44.50	III
		29	0.72	2.33	30.45	I			28	0.73	3.01	40.40	II
23	12	25	0.78	2.59	34.75	I	?	22	24	0.75	2.96	39.85	I
		27	0.80	2.57	34.51	II			26	0.72	3.06	40.10	II
									28	0.72	3.11	41.60	II
?	12	25	0.76	2.38	33.09	I	2479	22	24	0.78	2.21	31.64	I
		27	0.77	2.22	30.80	I			26	0.77	2.14	30.60	I
		29	0.74	2.41	33.44	I			27	0.75	2.18	31.20	I
46	14	24	0.69	3.37	43.41	III	1512	22	25	0.73	2.03	30.59	I
		26	0.73	2.69	34.54	I			28	0.70	2.09	31.03	I
		28	0.72	2.92	37.59	II							

Means: R.Q., 0.74±0.003; cal./kg./hr., 2.53±0.03; cal./sq.m./hr., 33.73±0.39.

To determine whether it is possible to repeat metabolism measurements after a fast of approximately 24 to 30 hours—that is, to determine whether during this interval the metabolic rate remains constant—numerous tests were run with the results shown in table 1. Examination of this table shows that

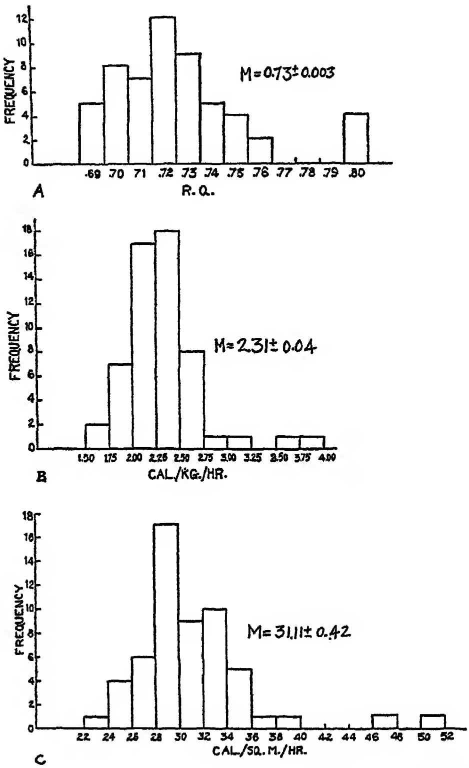

Fig. 2 The respiratory quotient and the metabolic rate of the hen 23 to 32 hours without food. Fifty-four tests on thirty-two White Plymouth Rock hens, 18 to 22 months old.

on the whole, if the activity of the bird is uniform, it is possible during this time to repeat with accuracy metabolic rate determinations on the same hen.

Combining the results on basal metabolism shown in figure 2, B and C, and table 1 (age of hens, 12 months or more), it appears that the average basal metabolism of the mature hen

is not far from 2.4 calories per kilogram per hour and 32.4 calories per square meter per hour, or 57.6 calories per kilogram per day and 778 calories per square meter per day.

The variability of the basal metabolism. The fifty-four basal metabolism tests summarized in figure 2 were made on

TABLE 2

The variability of the basal metabolism (White Plymouth Rock hens, 18 to 22 months old; non-laying)

HEN NO.	DATE OF TEST	WEIGHT	CAL./KG./HR.	CAL./SQ.M./HR.	CHANGE IN METABOLISM (CAL./SQ.M./HR.)	ACTIVITY
		kg.			%	
1594	11/ 2/31	2.26	2.41	31.62		I
	12/30/31	3.04	1.64	23.72	−25	I
1616	11/ 5/31	2.17	2.29	29.73		I
	12/30/31	2.84	1.83	25.95	−13	II
1549	11/ 5/31	2.08	2.57	32.77		I
	1/ 6/32	2.59	2.47	33.95	+ 4	I
1667	11/ 9/31	2.24	2.59	33.84		II
	12/ 1/31	2.40	3.81	51.20	+51	II
	1/ 6/32	2.77	2.36	32.23		I
	1/29/32	3.00	2.05	29.62	− 8	I
2510	11/12/31	2.43	2.38	31.90		I
	12/ 1/31	2.26	2.50	32.80	+ 3	I
1546	11/12/31	2.18	2.71	35.19		I
	1/ 8/32	2.97	2.03	29.18	−17	I
1516	11/17/31	2.65	1.97	27.14		II
	1/20/32	2.72	1.79	24.85	− 8	I
1659	11/20/31	2.56	2.06	28.14		I
	1/22/32	2.73	1.99	27.83	− 1	I
1615	12/ 1/31	2.80	1.74	24.54		II
	1/ 6/32	2.85	1.76	24.75	+ 1	II
2541	12/ 7/31	2.23	2.67	35.00		I
	2/ 3/32	2.58	2.11	29.00	−17	I
2510	1/20/32	2.27	2.18	28.68		?
	2/ 3/32	2.32	2.40	31.57	+10	I

thirty-two hens over a period of several months. Since more than one test was run on a large number of the hens, it is possible to arrange the results derived from these hens in such a way that the question of the variability of the basal metabolism can be studied. This is done in table 2. The data are from

hens that were not laying. It is evident that in several instances the change in the metabolic rate was large.

The heat loss due to vaporization of water. In this method of metabolism measurement, the water elimination of the subject is determined by the gain in weight of the sulphuric acid bottles coming in line immediately after the animal chamber. If during an experiment an egg is laid or feces are eliminated, the vaporization of water would be increased. The water elimination in such an experiment should therefore not be used in a study of the insensible water loss of the birds. Of

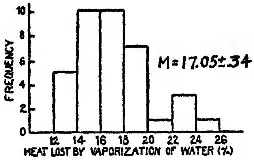

Fig. 3 The percentage of heat lost by vaporization of water. The data are from the basal metabolism studies summarized in figure 2.

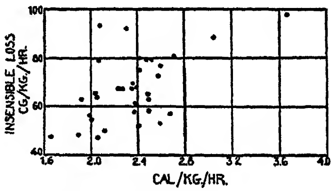

Fig. 4 The correlation between insensible loss and metabolic rate. The data are from the basal metabolism studies summarized in figure 2. The mean insensible loss is 0.675 gm. per kilogram per hour; the mean basal heat production, 2.32 calories per kilogram per hour. The correlation coefficient is 0.411.

the fifty-four tests depicted in figure 2, thirty-seven showed no egg or feces in the chamber. The average insensible water loss in these experiments was 0.68 ± 0.001 gm. per kilogram per hour. The minimum was 0.46, the maximum 0.99 gm. In each of the thirty-seven tests the percentage of heat lost by vaporization of water was calculated, 0.585 calories being assumed to be the amount of heat required to vaporize 1 gm. of water. The results are shown graphically in figure 3. It is evident that the distribution of the data is close to the normal.

Correlation between insensible loss and metabolic rate. It has been shown by Benedict and Root ('26) in man and by Kriss ('30) in cattle that there is a high positive correlation between the total insensible loss of the organism and the

TABLE 3

The effect of egg production on the fasting metabolic rate
(White Plymouth Rock hens, 18 to 22 months old)

HEN NO.	DATE OF TEST	WEIGHT	CAL./KG./HR.	CAL./SQ.M./HR.	CHANGE IN METABOLISM (CAL./SQ.M./HR.)	ACTIVITY	EGG PRODUCTION FOR MONTH IN WHICH THE TEST WAS MADE	DAYS BETWEEN WHICH OR ON WHICH EGGS WERE LAID
		kg.			%			
2321	11/ 2/31	2.32	2.41	31.90		II	11	12–31
	1/ 6/32	2.50	2.51	34.15	+ 7	II	13	12–30
2169	11/ 5/31	2.04	2.35	29.83		II	1	25
	12/30/31	2.23	2.50	32.74	+10	I	11	1–31
2197	1/22/32	2.65	2.15	29.45		?	3	8, 24, 27
1699	11/23/31	2.49	2.38	32.43		I	0	
	1/29/32	2.64	2.58	35.60	+10	II	4	6, 24, 25, 30
1453	12/ 1/31	2.28	2.68	35.33		II	0	
	2/ 3/32	2.48	2.78	37.60	+ 6	II	16	1–28
2440	11/ 9/31	3.11	1.91	27.88		I	1	28
	2/10/32	3.17	2.23	32.59	+17	I	13	2–26
1562	11/12/31	2.10	2.24	28.77		I	0	
	1/ 8/32	2.46	2.08	27.96	+ 3	II	0	
	2/11/32	2.44	2.85	38.23	+37	I	11	1–29
2479	12/18/31	2.81	2.02	28.40		I	0	
	2/17/32	2.94	2.18	31.15	+10	I	5	23–29
1512	11/20/31	3.24	2.04	30.17		I	0	
	1/22/32	3.28	2.02	27.83	− 8	I	0	
	2/19/32	3.27	2.06	30.81		I	5	20–28
	2/25/32	3.21	2.17	31.97	+ 4	I		
2504	2/25/32	2.80	2.20	30.95		I	3	24–27

metabolic rate. Many others have worked on the problem (Du Bois, '36, p. 62). A measurement of the total insensible loss being a necessary part of a metabolism test by the Haldane method, data were at hand for a determination of the relationship between the insensible loss and the metabolic rate.

The data are from the experiments incorporated in figure 2, and are therefore basal data. All insensible losses from experiments in which an egg or feces were found in the chamber were discarded for the purposes of this correlation. The correlation is shown by means of a scatter diagram (fig. 4). The correlation coefficient of the two sets of data is 0.411. Fisher's 't' test indicates that the correlation is significant.

The effect of egg production on the fasting metabolic rate. The metabolism tests forming the basis of figure 2 were made over a period of several months. Records of the egg production of the flock were kept during that time. Since several of the hens laid fairly well during the months in which their metabolic rates were determined, it is possible to assemble the data in such a way that the effect of egg production can be studied. This is done in table 3.

DISCUSSION

Mitchell and Haines ('27 b) and Benedict, Landauer and Fox ('32) have pointed out that a fast of at least 48 hours is necessary to bring the hen into the post-absorptive, and therefore the basal, condition. Under the conditions of the present experiments, it is clear that in the interval extending from 24 to 30 hours after food the metabolic rate of the hen is uniform (table 1) and the respiratory quotient is on the average about 0.73 (figs. 1A and 2A; table 1). It is therefore evident that further fasting is not required; and by analogy with man it appears justifiable to speak of the metabolic rate of resting hens without food for 24 to 30 hours as the basal metabolism. The average basal metabolic rate reported here is practically the same as the mean of the averages obtained by Mitchell and Haines ('27 b) and Benedict, Landauer and Fox ('32) in day experiments, and very close to the mean of the averages of the day and night experiments of Benedict, Landauer and Fox.

Figure 2 shows that several of the hens were distinctly aberrant with respect to the fasting respiratory quotient and the metabolic rate. It is worth noting that the hens with the high respiratory quotients were not the ones with the high

metabolic rates. The bird (no. 1667) showing the highest metabolic rate (51.20 calories per square meter per hour) showed a normal rate 3 weeks earlier, and 5 and 8 weeks later (table 2). This brings up the important question of the variability of the basal metabolism. The data in table 2 indicate a fairly constant basal metabolism in most of the hens, but there are several distinct exceptions. Although the majority of the hens gained in weight between metabolism tests, there does not appear to be any correlation between the increase in weight and any change in metabolic rate. The recent work of Benedict and Ritzman ('35) indicates that in dairy cattle individual animals may show very great lability of the basal metabolism. In the hen the lability is not as great as in dairy cattle but it is greater than in man (Du Bois, '36, p. 184).

An age effect on the basal metabolism seems evident from the data here reported. The metabolic rates shown in figure 1 (B and C) after a fast of approximately a day's duration are distinctly higher, on the average, than the rates shown in figure 2 (B and C), where the fast was of about the same duration. The hens in the latter group were 8 to 10 months older than those in the former. It appears therefore that the basal metabolism of the hen decreases with age. Experiments of Mitchell, Card and Haines ('27) and of Mitchell and Haines ('27 b) indicate that the basal metabolism of mature hens is lower than that of pullets, and the work of Brody, Funk and Kempster ('32) shows that the heat production of chickens declines with age.

The percentage of heat lost by vaporization of water in the fasting hen is on the average 17 (fig. 3). This is much lower than the average figure (49%) obtained by Benedict, Landauer and Fox ('32) on a fasting hen and cock. This difference is doubtless explainable by the fact that the average environmental temperature at which the measurements of Benedict and co-workers were made was several degrees higher than the average in the present series. In fact, Doctor Benedict in a private communication to the author states that in experiments on geese, made at a lower environmental temperature

than in their experiments on chickens, the percentage of heat lost by vaporization is much lower. The heat lost by vaporization in man is usually considered to be about 24% of the total heat loss (Du Bois, '36, p. 62), but Burton and Murlin ('35) have reported lower values; 17.8% on the average. The average in the present paper is very close to this.

According to Gerhartz ('14) the basal metabolism of the laying hen is definitely higher than that of the non-laying hen, and Mitchell and Haines ('27 b) report a small amount of evidence to support this view. On the other hand, Brody, Funk and Kempster ('32) did not note, in a limited amount of data, any marked difference between the heat production of good-laying and of poor-laying pullets. Examination of table 3 in the present paper shows in all instances an increase in the basal metabolic rate with the onset of egg production. However, in most hens the increase is small and in several it is of no significance. Nevertheless in at least two hens (2440 and 1562) the increase is large enough to be significant. Of importance also may be the fact that during the same period several of the non-laying birds in the flock showed definite declines in the basal metabolism (table 2). The data as a whole appear to justify the conclusion that egg production is accompanied by some increase in the basal metabolism. Further information is required, however, before a definite statement can be made.

SUMMARY

In a study of the energy metabolism of the hen (156 determinations on fifty-four birds) it was found that:

1. In prolonged fasting the metabolic rate fell progressively until about the seventy-fifth hour. From then on to about the 100th hour the rate appeared to be practically uniform. The respiratory quotient showed no great change after about the twenty-fourth hour (fig. 1).

2. The average basal metabolic rate of mature hens, after a fast of approximately 24 to 30 hours' duration, was not far from 2.4 calories per kilogram per hour and 32.4 calories per

square meter per hour (fig. 2, B and C; table 1), or 57.6 calories per kilogram per day and 778 calories per square meter per day. The average basal respiratory quotient of hens was about 0.73 (figs. 1A and 2A; table 1).

3. During the interval extending from 24 to 30 hours after food the metabolic rate in individual hens was uniform (table 1).

4. Most of the hens showed a fairly constant basal metabolism over a period of 1 to 2 months, but several showed great lability in the basal metabolism (table 2).

5. The basal metabolism was lower in older hens.

6. The heat loss due to vaporization of water (basal) varied from 12 to 25% of the total heat loss and averaged 17% (fig. 3).

7. The basal insensible loss and the basal metabolism showed a positive correlation (fig. 4).

8. Egg production was accompanied by a small increase in the basal metabolism (table 3).

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THE EFFECT OF YEAST ON THE LIVER GLYCOGEN OF WHITE RATS DURING HYPERTHYROIDISM

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It is generally accepted that a reduction of the liver glycogen in the cat, rat, mouse, guinea pig, or rabbit, can be brought about by feeding desiccated thyroid (Frazier and Friedman, '35; and others). Coggeshall and Green ('33) found that the liver glycogen in rats was reduced most by thyroxin, less by dried thyroid gland, and not at all by inorganic iodides, when these substances were administered in proportion to their iodine content.

In 1931, Himwich, Goldfarb and Cowgill found that dogs fed desiccated thyroid needed more vitamin B complex as judged by the onset of anorexia and loss of weight. This was confirmed in the pigeon by Cowgill and Palmieri ('33).

Sure and Smith ('34) reported that vitamin B (B_1), when fed simultaneously with oral or subcutaneous doses of thyroxin, showed a protective action against the effect of the thyroxin as judged by the loss of weight. Later Sure and Buchanan ('35) reported on the quantitative balance between thyroxin and vitamin B, in which they determined the minimal dose of vitamin B that would still produce a gain in weight while the animals were receiving thyroxin.

The question then arises: If vitamin B will prevent a loss of weight in rats receiving thyroxin, will it also prevent a loss of liver glycogen? Abelin ('30) and Abelin, Knochel and Spichtin ('30) found that large amounts of casein, egg yolk, or yeast in

the diet of hyperthyroid animals would lower the basal metabolic rate and would also prevent liver injury. In their experiment they fed 100, 200 or 300 mg. of thyroid per day for 6 days together with yeast and obtained beneficial results, even though the animals still lost weight. The vitamin content of the yeast is not stated.

It was thought advisable to examine the change in the percentage of liver glycogen, if any, when small amounts of thyroxin were injected daily over a longer period of time, instead of the short 6-day period used by Abelin et al. Yeast, in which the vitamins B and G are known, was to be fed in order to produce a constant weight, or a gain in weight, in rats receiving thyroxin, and the liver of these rats then analyzed for the percentage of glycogen and compared with the control rats.

EXPERIMENTAL WORK

In this experiment dry yeast [1] was used as the source of vitamin B instead of injecting crystalline vitamin B. This was done to eliminate any possible shortage of other vitamins in the vitamin B complex, for if we injected vitamin B and used autoclaved beef as a source of vitamin G we would not be sure that the animals would receive the other essential vitamins in the complex. The yeast used in the experiment contained 18 international units of vitamin B per gram and 20 Sherman units of vitamin G per gram. A daily injection of 0.1 mg. of crystalline thyroxin [2] was given subcutaneously. The following diet (no. 3) was used: Steenbock's no. 40 salt mixture, 4 gm.; cod liver oil, 2 gm.; Crisco, 10 gm.; casein (extracted with dilute acetic acid, followed by 60%, then 90% ethyl alcohol), 20 gm.; starch, 64 gm.

Group 1 (positive controls). These rats received a mixture of 97 gm. of diet no. 3 plus 3 gm. of yeast. This gives 54 international units of vitamin B and 60 Sherman units of vitamin G per 100 gm. of food. No thyroxin injections were given this group. Seven rats (five males, two females) were used. At

[1] Fleischmann's dried yeast, no. 15190.
[2] Squibb's no. 57999.

the beginning of the experiment two males were killed and the liver glycogen determined. The other three males and the two females were continued on the above diet until the end of the experiment. The rats ate 12 to 13 gm. per day. Water was constantly supplied.

Group 2 (test rats). Each rat received 10 gm. of diet no. 3 plus 3 gm. of dry yeast per day, giving each rat 54 international units of vitamin B and 60 Sherman units of vitamin G per day. The 13 gm. of food was consumed nearly every day. Nine rats (five males, four females) were used. Each rat also received 0.1 mg. of thyroxin injected subcutaneously per day. Water was constantly supplied.

Group 3 (negative controls). These rats received the same diet as group 1 plus a daily injection of 0.1 mg. of thyroxin subcutaneously. Eight rats (six males, two females) were used. The rats ate 12 to 13 gm. of food per day. Water was constantly supplied.

EXPERIMENTAL RECORD

9/9/36. The special diets and the daily injections were begun.

9/18/36. The yeast content of the negative and positive controls was reduced to 1.0 gm. of yeast plus 99 gm. of diet no. 3. Note that the reduction was made in both groups 1 and 3. This change was made because the negative controls were still gaining weight at this date. A balance of diet was sought so that group 1 would gain weight on the amount of yeast provided, and so that group 3 (negative controls) would lose weight on the same diet when injected with 0.1 mg. of thyroxin per day. It was then planned, by raising the yeast content, to produce a gain in weight in group 2 which was also receiving thyroxin.

9/21/36. Rat no. 89 in group 2 died. (See table 2.)

9/22/36. Rat no. 88 in group 3 died. (See table 3.)

9/27/36. The weights of the negative controls had dropped varying degrees. The positive controls and the test rats continued to gain in weight. All of the rats were killed and the liver glycogen determined by a modification of the Pfluger

method by Good et al. ('33). The sugar obtained in this analysis was then determined by the method of Shaffer and Somogyi ('33). Forsgren ('29) has shown that there is a functional variation in the liver glycogen content. All of the rats were therefore fed at the same time each day to produce the same cycle of glycogen variation in each rat; and the rats were all killed 24 hours after the last injection and feeding and all of the livers were dissected from the rats within 1 hour, limiting, therefore, any cyclic variation in the glycogen to a minimal change.

TABLE 1

Group 1 (positive controls)

RAT NO.	SEX	WEIGHT AT START OF EXPERIMENT	WEIGHT AT END OF EXPERIMENT	LIVER GLYCOGEN
				%
73	M	Both rats killed at beginning		2.17
74	M	of experiment		2.34
		gm.	*gm.*	
76	M	144	177	3.74
83	M	143	162	2.63
84	M	151	185	2.67
80	F	120	139	2.56
81	F	99	138	2.50
			Average	2.65

RESULTS

The rats in group 1 (positive controls) all made a gain in weight. The values for the liver glycogen are normal and check with one another. Table 1 shows the weights of the rats and the results of the analysis.

The test rats in group 2 had not lost weight by the end of the experiment. Three of the rats remained constant in weight (nos. 86, 87, 90) and the other five rats made a considerable gain in weight. All of the liver glycogen percentages are normal in range, the average being slightly below the rats of group 1. The results of the analysis and the weights of the rats are shown in table 2.

The rats in group 3 (negative controls) received the same diet as group 1, which contained enough yeast to produce a

steady gain in weight, as evidenced by the growth of the rats in group 1. The rats in group 3 were then made to lose weight by the injection of 0.1 mg. thyroxin per day. Two rats (nos. 82 and 57) did not have a loss of weight at the end of the experiment, although their weight fluctuated during the experiment. The percentage of these two rats is close to the normal values in table 2. The results obtained on these two rats are therefore discarded and the negative control values are based on the remaining five rats, all of which show the low liver glycogen percentages of hyperthyroidism. The results of this group are

TABLE 2

Group 2 (test rats)

RAT NO.	SEX	WEIGHT AT START OF EXPERIMENT	WEIGHT AT END OF EXPERIMENT	LIVER GLYCOGEN	COMMENT
		gm.	*gm.*	%	
86	M	164	163	2.41	Weight remained constant
87	M	154	154	2.29	Weight remained constant
90	M	166	165	1.86	Weight remained constant
89	M	156	106	...	Died 9/21/36
58	F	115	125	1.99	Gain in weight
59	F	123	142	2.29	Gain in weight
78	F	113	137	2.23	Gain in weight
79	F	107	119	2.39	Gain in weight
92	M	140	156	2.02	Gain in weight
			Average	2.18	

tabulated in table 3. In table 3 the column headed 'Highest weight recorded' means the highest weight that these rats showed, before the sudden effects of the injections caused hyperthyroidism and subsequent loss of weight. Group 2 was protected against the hyperthyroidism by the slightly higher vitamin content of the diet, and no high-point in the weight is then obtainable, because the rats made a constant gain in weight.

Table 4 is included to show the effect of severe hyperthyroidism on the liver glycogen of rats, when fed 0.1 gm. of thyroid gland each day for 12 days. Table 5 shows more normal values for the liver glycogen in rats which were in the same series as those in which severe hyperthyroidism was produced (table 4).

The rats in group 1 (positive controls) receiving 2.1 to 2.4 international units of vitamin B and 2.4 to 2.6 Sherman units of vitamin G per day, gained weight. The negative controls in group 3, receiving the same amount of vitamin, lost weight when injected with thyroxin and glycogen per cent in the liver was less than in group 1. In the test rats (group 2), each rat

TABLE 3

Group 3 (negative controls)

RAT NO.	SEX	WEIGHT AT START OF EXPERIMENT	HIGHEST WEIGHT RECORDED	WEIGHT AT END OF EXPERIMENT	LIVER GLYCOGEN	COMMENT
		gm.	gm.	gm.	%	
60	M	151	155	136	0.68	
75	M	131	137	113	0.83	
77	M	132	143	127	1.14	
85	M	131	152	123	0.28	
88	M	142	152	117	...	Died 9/22/36
91	M	130	132	123	1.27	
57	F	105	113	111	1.63	Weight remained constant Results not averaged (see text)
82	F	111	119	119	1.56	
				Average	0.84	

TABLE 4

Severe hyperthyroidism

RAT NO.	SEX	WEIGHT AT START OF EXPERIMENT	WEIGHT AT END OF EXPERIMENT	LIVER GLYCOGEN
		gm.	gm.	%
47	M	156	113	0.16
46	M	172	131	0.18
48	F	145	115	0.26
55	M	171	154	0.06

TABLE 5

Normal rats (concurrent with table 4)

RAT NO.	SEX	DATE KILLED	LIVER GLYCOGEN
			%
50	F	All rats were killed 6/27/36 and had made a steady gain in weight before death.	2.95
51	F		2.05
53	M		1.63
56	M		1.89

received about 54 units of vitamin B and about 60 units of vitamin G per day and did not lose weight or liver glycogen when injected with the same amount of thyroxin as group 3. This demonstrates that when yeast is fed in an amount sufficient to prevent a loss of weight in hyperthyroid rats, a loss of liver glycogen can also be prevented, keeping the percentage of liver glycogen very close to that of normal animals.

DISCUSSION

In the literature a number of conflicting papers have appeared on the relationship between the vitamin B complex and liver glycogen. Ariyama ('33) found that the liver glycogen of pigeons increased during vitamin B deficiency, but under fasting conditions the liver glycogen was less than normal. Abderhalden and Wertheimer ('32) found an accumulation of glycogen in the liver during vitamin B deficiency. Westenbrink ('33) reported that the liver could form glycogen normally during vitamin B deficiency.

On the other hand, Labbé and associates ('33 a) found that vitamin B favors the deposition of glycogen in the liver and reduces glucemia and glycosuria. They also reported ('33 b) that the vitamin B complex favored the formation of glycogen in the liver, and also increased the glutathione content of the liver. Sure and Smith ('32) using rats that received the same number of calories as the vitamin B complex deficient rats, found a lowered liver glycogen in the vitamin deficient rats. Collazo and Bayo ('31) found a lowered glycogen content in B avitaminosis and also that yeast autolyzate would cure these pathological conditions, whereas insulin would produce the same beneficial results as the yeast without, however, curing the vitamin deficiency. He therefore concluded that the nutritional disturbances of vitamin B avitaminosis are not due to a pancreatic dysfunction, but are related to carbohydrate metabolism. Lajos ('36) found that vitamin B aids the storage of sugar as liver glycogen and acts similarly to insulin, but more slowly, in reducing blood sugar. The weight of evidence appears to be in favor of the view that vitamin B aids the storage of glycogen in the liver. This is substantiated by the work

of Abelin and associates and by the experiment performed above.

Westenbrink and Overbeek ('33) report that in the absence of vitamin B the resorption of glucose is lessened, and Osuka ('31) found that in normal animals yeast only promotes glycogen formation if the diet contains 1 to 2 gm. of sugar per day. Frazier and Friedman ('35) found a lower percentage of glycogen in the liver during hyperthyroidism, which could not be raised by the feeding of sugar.

If the lowering of the liver glycogen by vitamin B deficient diets is substantiated in future research, it is suggested that the loss of glycogen in the liver during hyperthyroidism might be due to the destruction (or loss of use) of the vitamin B (or B complex) by thyroxin or desiccated thyroid. The low liver glycogen might therefore be due to vitamin B deficiency; the vitamin B deficiency being brought on by the hyperthyroidism. This would be borne out by the work of Sure and associates in stopping a loss of weight, in rats receiving thyroxin, with vitamin B, and by Abelin et al., and by this experiment on liver glycogen during hyperthyroidism. It would also explain why Frazier and Friedman could not raise the liver glycogen during hyperthyroidism with sugar; because the vitamin B (or B complex) was destroyed or its function inhibited by the thyroid hormone.

CONCLUSIONS

1. A group of rats on a normal diet containing 2.1 to 2.4 international units of vitamin B (B_1) per day and 2.4 to 2.6 Sherman units of vitamin G per day gained in weight and showed normal liver glycogen values.

2. A group of rats on a normal diet containing 2.1 to 2.4 international units of vitamin B and 2.4 to 2.6 Sherman units of vitamin G and receiving 0.1 mg. of thyroxin subcutaneously per day, eventually lost weight and showed low liver glycogen values.

3. A group of rats on a normal diet containing 54 international units of vitamin B and 60 Sherman units of G and

receiving 0.1 mg. of thyroxin subcutaneously per day, still gained or remained constant in weight and showed normal liver glycogen values.

4. The change in the vitamin content of the diet of the test rats, as regulated by the amount of yeast, is responsible for the normal liver glycogen value.

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EFFECT OF ADDING COPPER TO THE EXCLUSIVE MILK DIET USED IN THE PREPARATION OF ANEMIC RATS UPON THEIR SUBSEQUENT RESPONSE TO IRON

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TWO FIGURES

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The necessity of copper as a supplement to iron for hemoglobin synthesis was first demonstrated by Hart et al. ('28). Since that time the mode of action of copper has been investigated (Joseph, '32; Elvehjem and Sherman, '32) and it has been shown that copper does not affect the assimilation of iron but functions in the conversion of iron to hemoglobin. Thus stored iron can only be utilized for synthesis of hemoglobin when copper is available.

In the recommended procedure for the experimental production of anemic rats for use in studies on availability of iron, however, this effect of copper has received no consideration. Rats are used which have been placed upon a whole milk diet until the hemoglobin concentration of the blood falls to a certain low level (2 to 5 gm. per cent) at which time exhaustion of iron reserves is taken for granted. The hemoglobin regeneration resulting from the subsequent feeding of a food is then taken as a measure of the amount of available iron in that food.

Elvehjem has stressed the necessity for exhaustion of body iron reserves in preparation of the test animals for studies of availability of iron in foods, for unless this is done, it is obvious that the hemoglobin synthesis which follows the

feeding of a food will be a measure of the copper content of that food as well as its available iron. Schultze and Elvehjem ('33) have reported that a "slight initial rise of hemoglobin and erythrocytes" results from the feeding of copper alone to anemic rats.

It seems logical to believe that a fall in hemoglobin to a low level in rats on a whole milk diet may occur even in the presence of tissue iron reserves, because of lack of adequate copper to catalyze its conversion into hemoglobin, and therefore that low hemoglobin values in animals on a milk diet regime can only be used as criteria of exhaustion of this store of iron if ample copper has been provided to insure its utilization. Accordingly, the effect of adding copper to the milk diet used for production of experimental nutritional anemia has been further investigated and the response to iron supplementation of rats made anemic in the presence of copper has been measured.

EXPERIMENTAL PROCEDURE AND RESULTS

Following the essentials of the Elvehjem-Kemmerer technic ('31) for the production of anemia, rats were weaned at 3 weeks of age and continued upon whole milk as their sole food. It has been previously found that the hemoglobin of rats handled in this fashion in this laboratory reached a concentration of 4.5 gm. per 100 cc. or below in 4 to 5 weeks. In these experiments, therefore, at the end of the fourth week each rat was given 0.05 mg. copper as copper sulfate daily for 1 to 2 weeks in addition to the whole milk diet in order to insure the presence of ample copper for the conversion of all residual iron into hemoglobin, and graded portions of iron as ferric chloride supplemented with copper and manganese were then given. The gains in hemoglobin of these rats in the following 6-week test period were measured and compared with the hemoglobin regeneration of rats which received the same amounts of iron but which were not given supplemental copper in the anemia preparation period. Details of the housing and care of these rats, method of giving

the supplements and hemoglobin measurement have been describd in a previous paper (Smith and Otis, '37 a).

Figure 1 shows graphically the effect of adding copper alone to the milk diet of anemic rats. Whereas, the hemoglobin concentration in the blood of the milk fed rats which were not given copper supplements fell steadily, the mortality being high beyond the eighth week, it may be seen that the addition of copper resulted in an increase in hemoglobin. The initial rise in hemoglobin concentration was, however, usually followed by a fall to approximately the original level and the decline in hemoglobin thereafter was slight, if any. This

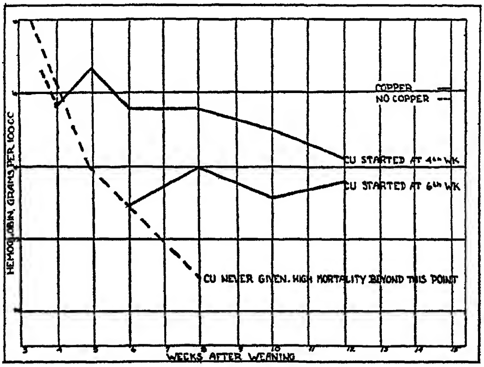

Fig. 1 Hemoglobin curves showing the effect of adding copper to the whole milk diet of anemic rats (average of ten males).

rise in hemoglobin occurred when the copper addition was made at the fourth week after weaning, when the hemoglobin level in these animals approximated 5 gm. per 100 cc. and also when the addition was made at the sixth week when the hemoglobin concentration has fallen to approximately 3.5 gm. per cent. Thus in both cases a body store of iron which became available for hemoglobin synthesis on the addition of copper was indicated, in spite of the low blood hemoglobin level characteristic of severe anemia.

These findings are in keeping with those of Schultze and Elvehjem ('33) referred to before, and substantiate their suggestion that "even severely anemic rats contain a small

amount of iron which may be used for hemoglobin building in the presence of adquate amounts of copper.'' Our attention has recently been called to the work of Hubbell ('36) who found that the feeding of copper to anemic rats (4 to 5 gm.

TABLE 1

Hemoglobin regeneration in anemic rats at different levels of iron intake. Comparison of hemoglobin gains in rats made anemic in the presence or absence of copper

AMOUNT OF Fe FED DAILY	RATS MADE ANEMIC IN ABSENCE OF COPPER				RATS MADE ANEMIC IN PRESENCE OF COPPER			
	Number of rats	Initial hemoglobin	6 weeks gain hemoglobin	± P.E.	Number of rats	Initial hemoglobin	6 weeks gain hemoglobin	± P.E.
	Males				Males			
mg.		*gm./100 cc.*	*gm./100 cc.*			*gm./100 cc.*	*gm./100 cc.*	
0.000	6	4.3	0.30		8	5.0	—0.60	0.20
0.014	14	4.0	1.10	0.18	10	5.5	0.50	0.11
0.050	20	4.0	3.50	0.14	12	5.6	1.40	0.21
0.071	5	3.9	4.30	0.24	13	4.8	2.80	0.28
0.100	14	4.0	5.50	0.14	13	4.8	3.00	0.28
0.150	11	4.0	8.20	0.21	13	4.9	5.40	0.36
0.200	12	3.8	9.10	0.19	10	4.1	5.70	0.29
0.250	12	3.5	10.40	0.18	8	4.0	6.90	0.40
0.300	7	4.1	10.10	0.29	10	3.9	7.90	0.23
0.400					1	3.9	9.60	
0.500					3	3.4	10.50	
	Females				Females			
0.000	12	6.0	0.10		12	6.0	—0.42	0.16
0.014	5	4.1	2.30	0.32	12	6.4	0.71	0.25
0.050	13	4.4	4.20	0.33	11	5.8	2.08	0.37
0.071	15	3.9	5.60	0.25	11	5.9	2.75	0.30
0.100	12	4.0	6.50	0.15	10	5.9	4.50	0.23
0.150	10	3.7	9.50	0.26	10	6.2	5.70	0.39
0.200	8	3.7	10.90	0.25	8	5.5	6.10	0.37
0.250	6	4.1	10.70	0.18	11	5.2	7.40	0.31
0.300	7	4.6	10.20	0.16	9	4.0	8.40	0.35
0.400					5	3.8	10.10	
0.500					2	3.8	10.70	

per cent) caused an increase in hemoglobin of from 0.4 to 1.5 gm. She states, however, that "these slight increases were within normal variability in hemoglobin and did not indicate any necessity for administration of copper during the preparation period to insure full exhaustion of iron reserves.''

That the iron reserves in anemic rats whose hemoglobin level has fallen to a low level, though relatively small, does have an appreciable effect upon the subsequent response of these rats to iron supplementation is quite apparent from our experiments, however. Comparative hemoglobin responses to iron and copper supplementation of rats made anemic in the presence or absence of copper in the foreperiod are tabulated in table 1 and shown graphically in figure 2. At all levels of iron feeding, the rats which had not received copper in the anemia preparation period regenerated more

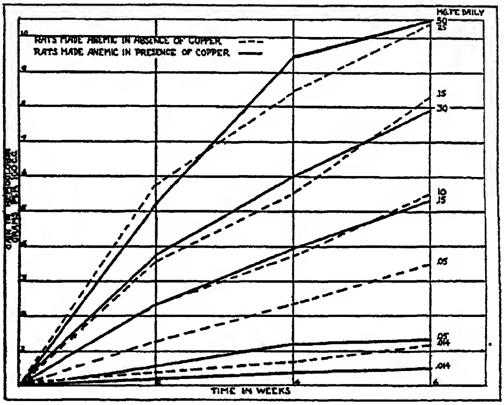

Fig. 2 Hemoglobin curves showing difference in response to iron supplementation between rats made anemic in the presence of copper and those given no copper in the anemia preparation period.

hemoglobin than those which had been made anemic on the whole milk diet supplemented with copper from the fourth week on.

For example, the copper-in-foreperiod rats given 0.05 mg. of iron as ferric chloride daily plus 0.05 mg. copper as copper sulfate gained only 1.4 gm. of hemoglobin per 100 cc. blood in the 6-week test period, whereas the rats receiving the same iron and copper supplements, but which were not made anemic in the presence of added copper showed an average hemoglobin regeneration of 3.5 gm. per cent in a test period of the same length.

Again, it may be seen that when rats have been made anemic in the presence of copper 0.3 mg. of iron as ferric chloride, adequately supplemented with copper and manganese, given daily did not raise the hemoglobin to the normal level in a 6-week test period.

Thus, in every case, the gains in hemoglobin of the anemic rats which had not received copper in their preparation period were abnormally large and therefore cannot be considered a true measure of their response to the iron supplementation. It would seem, therefore, that unless rats were made anemic in the presence of sufficient copper for the complete utilization of iron reserves, their use for measurement of available iron in foods may give erroneous results.

A difference in response between males and females as previously reported (Smith and Otis, '37 b) may still be observed, hemoglobin gains being greater in the female at the same level of iron. However, the sex difference observed is of smaller magnitude when copper is given in the last 2 weeks of the foreperiod. This fact strongly indicates that part, if not all, of this sex difference is due to a greater store of iron in the female of the same hemoglobin concentration, and consequently a greater difficulty in depletion, rather than to a difference between males and females in their ability to utilize the iron supplements. A longer period of copper feeding to insure complete absence of residual iron might be expected therefore to erase all sex differences in response to iron.

It is interesting to note that although the hemoglobin concentration in the females receiving copper in the foreperiod was higher at the beginning of the test period than that of the females receiving no copper, their response to iron and copper supplementation was much less. This fact gives further evidence for our belief that a low hemoglobin concentration cannot safely be used as criterion of the absence of residual iron, for the store was obviously less in the females receiving copper in spite of their higher hemoglobin concentrations.

CONCLUSIONS

Rats made severely anemic upon a whole milk diet according to the Elvehjem-Kemmerer technic contain residual iron which is converted into hemoglobin when copper alone is administered in adequate amounts. Animals which do not have their iron reserves depleted in the presence of copper regenerate much more hemoglobin in response to subsequent iron and copper supplementation than do rats which have been given sufficient copper in the preparation period for complete utilization of iron stores.

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ON THE IDENTITY OF THE GOLDBERGER AND UNDERHILL TYPES OF CANINE BLACKTONGUE. SECONDARY FUSO-SPIROCHETAL INFECTION IN EACH [1, 2]

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ONE FIGURE

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Chittenden and Underhill ('17) described a new experimental disease of dogs characterized by anorexia, weight loss and a rapidly progressive inflammatory process in the mucus membranes of the mouth. The condition was produced by feeding a simple diet, adequate in proteins and calories but deficient in vitamins A and D. In 1923, Goldberger and Wheeler ('28) produced a similar disease by feeding a different diet, deficient in 'pellagra preventive' factors but otherwise adequate for nutrition. This picture was identified with canine blacktongue, a disease frequently recognized by southern veterinarians, and was named 'experimental blacktongue.' It could be cured by the addition of yeast or red meat to the diet.

[1] This is no. 7 in a series of coordinated studies on pellagra in man and associated deficiency diseases of animals from the Departments of Physiology, Pathology, Bacteriology and Medicine of Duke University with the coperation of Dr. Y. Subbarow of the Department of Biochemistry of Harvard Medical School. Previous publications are listed in the 'Literature Cited' as Smith ('32), Ruffin and Smith ('34), Smith and Sprunt ('35), Dann ('36), Ruffin and Smith ('37) and Smith and Ruffin ('37).

[2] The expenses of this work were covered in part by a grant from the American Cyanamide Company of New York and the Lederle Laboratories, Pearl River, N. Y.

[3] Aided by the Henry Strong Denison Medical Foundation.

Underhill and Mendel ('28), recognizing that a similar experimental disease appears as a result of two different deficient diets, reported very extensive experiments. They were able to produce their disease on diets containing large amounts of red meat and yeast. No adequate explanation has ever been offered for the striking similarity of the syndromes resulting from such dissimilar diets.

Since both these syndromes were characterized by fetid breath and gangrene of the mouth, it occurred to us that this similarity might be due to an identical infection secondary to different nutritional deficiencies. Goldberger's experimental blacktongue and the Underhill syndrome have been produced in this laboratory and the type of secondary infection studied.

EXPERIMENTS WITH THE GOLDBERGER TYPE OF CANINE BLACKTONGUE

Three dogs were fed Goldberger's diet no. 268 (Goldberger and Wheeler, '28) without modification, and the clinical picture of blacktongue was produced, with death in 94 to 107 days. For further study Goldberger's diet no. 123 [4] was modified by substituting Underhill and Mendel's more complete salt mixture ('28) for the sodium chloride and calcium carbonate, yellow corn meal for the white corn meal, and by doubling the amount of cod liver oil. The commercial casein (Merck) was leached with acidulated water and extracted with 95% alcohol.[5] The diet used consisted of yellow corn meal, 400 gm., coarsley ground California black-eyed

[4] This diet was recommended by Dr. W. H. Sebrell, U.S.P.H.S., P.A. Surgeon, in charge of F. S. Nutrition, Washington, D. C.

[5] Method: 25 pounds of commercial casein are treated with 30 liters of distilled water acidified with 0.2% glacial acetic acid (60 cc.). The mixture is hand stirred frequently over a 48-hour period with constant agitation maintained by a stream of air led to the bottom of the 12½-gallon container. The acidified water is then poured off through a multilayered gauze screen. The water is absorbed, to promote drying, and further extraction carried on during the next 48 hours by adding 20 liters of 95% alcohol, with frequent stirring. The alcohol is decanted as above. The casein is then spread thinly over a large surface area to dry. Drying may be speeded up by currents of air as generated by electric fans.

peas (Vigna sinensis) 50 gm., casein 60 gm., sucrose 32 gm., cottonseed oil 30 gm., cod liver oil 30 gm., and salt mixture 12 gm. This supplied a total of 2535 calories. The cornmeal, cow peas and salt mixture were diluted with 3 volumes of water and cooked for 2 hours in an open steam cooker. After cooking, the other ingredients were added and thoroughly mixed. This food was fed ad libitum and a minimum of 125 calories per kilogram of body weight was offered each day.

Using this diet, canine blacktongue was produced ninety-six times in fifty-three different adult dogs of various breeds and both sexes. In the acute attack, smears and dark field preparations from the mouth lesions showed the constant presence of large numbers of fuso-spirochetal organisms. These organisms may be found in small numbers about the teeth in normal dogs (Smith, '32). In our experience, they always increase enormously in numbers during the acute stage of blacktongue and almost disappear after recovery induced by dietary treatment (Harvey et al., unpublished data) only to reappear with the next attack.

Material for culture and animal inoculation was obtained from the mouth of a dog in a severe attack of blacktongue. Saliva, containing mucus and small pieces of necrotic tissue, was aspirated from the dog's mouth with a sterile pipette. Smears of the material were stained by Gram's method, gentian violet, and Fontana's silver nitrate and preparations were studied with the dark field apparatus. The following organisms were tentatively identified: Treponema microdentium, Treponema macrodentium, Treponema vincenti, Treponema buccale, Leptospira trimerodonta, Spirillum sputigenum, fusiform bacilli, vibrios, and gram positive cocci, singly, in pairs, and chains (fig. 1). No spore bearing organisms were found by smear or culture. Two guinea pigs were inoculated in the groin with 1 cc. and two with 2 cc. of the fresh material from the dog's mouth. After 6 to 10 days all four animals developed abscesses in the groin which apparently contained all the original organisms except the Leptospiras. Anaerobic cultures from these abscesses were

also negative for spore bearing organisms. Similar abscesses were produced in the groins of other guinea pigs by inoculating 0.25 cc. to 0.5 cc. of the undiluted pus. Pus obtained from the third serial transfer of the infection in guinea pigs was introduced into the lungs of four normal dogs through a bronchoscope.[6] One dog escaped infection, one died in 48

Fig. 1 Drawing (× 1000) of a dark field preparation made from the saliva of a dog with the Goldberger type of blacktongue. The small tightly coiled spirochetes in the left upper quadrant of the drawing are morphologically identical with Treponema microdentium (Noguchi). The larger tightly coiled spirochetes at the top of the drawing resemble Treponema macrodentium (Noguchi). The thin irregularly coiled spirochetes in the center are Treponema Vincenti (Blanchard). The thick spirochetes showing double centers near the bottom of the drawing are apparently Treponema buccale (Luhe) (Dobell). In the upper right segment are leptospiras which may be Leptospira trimerodonta (Hoffmann). Scattered about in the field are fusiform bacilli which may be recognized by their characteristic shape. The small comma-shaped organisms are vibrios and the larger curved organisms are spirilla resembling Spirillum sputigenum (Miller). Cocci, singly, in pairs, and short chains are always present.

The leptospiras are not always found but the other organisms are constantly present in varying proportions in this type of infection.

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[6] We wish to thank Dr. W. W. Eagle, of the Department of Surgery, in charge of Otolaryngology, for his assistance in introducing this material into the dogs' bronchi.

hours with an extensive pneumonitis, the other two died in 8 to 10 days with pulmonary abscesses of the fuso-spirochetal type. The organisms found in all these lesions were morphologically identical with those present in noma, Vincent's angina, and pyorrhea in man.

EXPERIMENTS WITH THE UNDERHILL SYNDROME IN DOGS

The diet used by Underhill and Mendel in their experiment no. 18 ('28) was fed to four dogs. The diet consisted of raw beef, cracker meal, cottonseed oil, yeast and Underhill and Mendel's salt mixture. In two additional dogs a part of the cottonseed oil was replaced by cod liver oil to supply 3.3 gm.[7] per kilogram of body weight per day. The animals which did not receive cod liver oil developed the typical syndrome of weight loss and progressive buccal necrosis and died after 54, 66, 81 and 133 days respectively. The two dogs which received cod liver oil remained well and were returned to stock after 152 days, which was 36 days longer than the maximum time required by Underhill and Mendel to produce the disease. These results indicate that the cod liver oil, used by us, contains a preventive factor.

The possibility that a deficiency of either vitamins D or C, or both, might contribute to the development of the syndrome prompted us to repeat the experiment, supplying 600 units of vitamin D, in the form of viosterol, and 1 mg. of ascorbic acid per kilogram of body weight per day. Four animals developed the characteristic syndrome on the Underhill and Mendel diet supplemented by vitamins C and D but not by cod liver oil, and died after 30, 76, 129 and 130 days. Two control dogs were fed as in the previous experiment receiving 3.3 gm. of cod liver oil per kilogram per day but no viosterol or ascorbic acid. One lived 137 days without the appearance of any mouth lesions and died of hook worm infestation, the other remained well for 156 days and was returned to stock.

[7] Mead-Johnson cod liver oil. One gram contained 1750 U.S.P. units of vitamin A and 175 units of vitamin D.

These experiments indicate that the Underhill syndrome can be prevented by some factor in the cod liver oil, and that this factor is not vitamin D. Underhill and Mendel ('28) could prevent or cure the disease with yellow butter, carrots or carotin, but not with cod liver oil. It seems probable that vitamin A is the significant factor and that the cod liver oil which they used contained fewer vitamin A units than the product used at present.

The use of these entirely different deficient diets in the same laboratory has given us convincing evidence that the clinical pictures of Goldberger's experimental blacktongue and the Underhill syndrome are identical. The bacteriologic findings were also identical. Smears and dark field preparations showed the presence of the same variety of organisms previously described (fig. 1). Material from the mouth of one of the dogs was inoculated into the groin of a guinea pig and the animal developed a typical fuso-spirochetal abscess after 4 days. The infection was successfully passed through four series of guinea pigs. Aerobic and anaerobic cultures were negative for pyocyaneus and gas gangrene organisms.

DISCUSSION

Fuso-spirochetal organisms were demonstrated in enormous numbers in the oral lesions occurring in dogs with each of the two types of dietary deficiency studied. There has been lengthy discussion as to the pathogenicity of the fuso-spirochetal group of organisms. The literature on this subject has been extensively reviewed elsewhere (Smith, '32), but the experiments reported here show that the group of organisms obtained from the mouths of the sick dogs is capable of producing severe infection in normal animals when the inoculation is made with minimal trauma. There is evidence (Smith, '30; Proske and Sayers, '34; Proske, '34) of a true symbiosis of these organisms as the causative factor of Vincent's angina, pyorrhea and fuso-spirochetal disease of the lungs.

Since Goldberger's experimental blacktongue is curable by yeast and meat, but not by vitamin A (Goldberger and Wheeler, '28) while the Underhill diet contains yeast and meat, the discovery of identical pathogenic groups of microorganisms in the very similar buccal lesions must be regarded as significant. In this connection it is important that Wallace, Wallace and Robertson ('33) reported the production of the same type of canine mouth lesions on diets adequate for nutrition. The lesions appeared after daily intravenous injections of one-fourth the lethal dose of scillaren B, a squill glucoside. 'Typical smears of Vincent's angina' are reported but no mention is made of organisms other than fusiform bacilli and spirochetes. Inoculation of pus from the lesions into the gums of normal dogs failed to reproduce the disease. The gross and microscopic pathology was described as very similar to that in human cases of Vincent's angina.

Miller and Rhoads ('35) observed the presence of fuso-spirochetal organisms in the oral lesions of dogs developing blacktongue on the Goldberger diet. They were unable to infect normal dogs by inoculating masses of these organisms into and under the labial mucus membrane. In our experiment we succeeded in producing infection in guinea pigs by inoculating large amounts of the material into the loose areolar tissue in the groin where the organisms find a favorable place for development.

It apears, then, that organisms of the fuso-spirochetal group which are normally present in the dog's mouth may, in at least one type of drug intoxication and two different types of nutritional disturbance, produce a necrotizing infectious process similar to that observed in spontaneous canine blacktongue. Recognition of the fact that entirely different factors may lower tissue resistance, and result in infection with the same group of organisms, should not vitiate the results of adequately controlled nutritional studies in which the secondary lesions are used as an indication of nutritional status.

At the same time, this concept furnishes a long-sought explanation for the similarity of the Underhill and the Goldberger syndromes.

These findings may have some bearing on problems of human nutrition. Fuso-spirochetal organisms are found almost invariably in the oral, and frequently in the vaginal, lesions of patients with acute pellagra. This association, well known in the South, has also been observed in Cleveland by Blankenhorn and Spies ('36); Spies, ('35) in alcoholic pellagrins. Sambon ('19) and Greenbaum ('27) have found the same organisms in the skin lesions of pellagra. Fuso-spirochetal infection in the mouth of a pellagrin disappears after treatment with materials potent in the pellagra-preventive factor. The Goldberger type of blacktongue is cured by the same materials (Harvey et al., unpublished data), indicating that this is the type of blacktongue which is analogous to pellagra.

CONCLUSIONS

1. The Underhill syndrome appears in dogs receiving meat and yeast and is prevented by adequate amounts of cod liver oil, whereas the Goldberger type of blacktongue appears in dogs receiving adequate amounts of cod liver oil, and is prevented by meat or yeast.
2. The Underhill syndrome in dogs is clinically and bacteriologically identical with Goldberger's experimental canine blacktongue.
3. The oral lesions in both the Goldberger and Underhill types of canine blacktongue are the result of infection with the fuso-spirochetal group of organisms secondary to lowered tissue resistance.
4. Dogs on the Goldberger diet may be used with confidence in assaying the pellagra curative value of various substances, because the appearance of secondary fuso-spirochetal infection is a reliable indicator of the nutritional status.

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IMMATURITY OF THE ORGANISM AS A FACTOR DETERMINING THE FAVORABLE INFLUENCE OF LACTOSE ON THE UTILIZATION OF CALCIUM AND PHOSPHORUS

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TWO FIGURES

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The literature concerning the influence of lactose on the utilization of calcium and phosphorus is full of contradictions which as yet have not been explained. Some of these studies have been made as part of an investigation of metabolism as affected by the parathyroid glands, others in relation to growth, and still others in connection with the effect of diet on intestinal flora.

From the work of Dragstedt and Peacock ('23) and Inouye ('24), it appears that lactose, in contrast to sucrose, dextrin and glucose, has the remarkable property of preventing tetany in the thyro-parathyroidectomized dog. Inasmuch as tetany is characterized by a fall in the level of blood calcium and a retention of phosphorus, it is reasonable to believe that lactose in some way influences favorably the body's metabolism of calcium.

Lactose feeding has been shown to bring about changes in the pH of the contents of the whole intestinal tract below the duodenum (Robinson and Duncan, '31) and to favor the development of an acidophilic flora (Rettger and Cheplin, '23). The bacteriological studies of Cannon and McNease ('23) and Cruickshank ('28) might be cited as favoring the view that any effect of lactose on calcium metabolism is a specific one and not the result merely of an increased solubility of calcium

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salts in the more acid medium. This view receives further support by the experiments of Kline, Keenan, Elvehjem and Hart ('32) with young chicks subsisting on a rachitic ration.

It is pertinent to point out that the negative results of Gross ('27) were obtained on mature dogs, whereas the positive findings reported by Bergeim ('26) for rats, Robinson, Huffman and Mason ('29) for calves, and Kline, Keenan, Elvehjem and Hart ('32) for chicks were obtained from immature organisms. Is it possible that immaturity in some way plays a definite role?

In view of the conflicting results cited, it seems obvious that the degree of influence of lactose on calcium and phosphorus utilization, if it exists, must be small. One might expect the slight effects of added lactose to be magnified when the diet contains only very small amounts of either calcium or phosphorus. In the experiments here reported the study of the effects of lactose was made under conditions where the amounts and ratios to each other of calcium and phosphorus have been carefully controlled.

In the course of this work, in which balance studies were made on three female dogs, definitely positive effects of lactose on calcium and phosphorus retention were noticed with two of the animals and no effect whatsoever with the third dog. In seeking for an explanation of this result, we were led to consider the obvious fact that the two animals yielding favorable results were much younger and immature in contrast to the third dog which was quite old. The previous history of these animals was unknown. Therefore we are unable to describe in any really precise terms the 'degree of immaturity' characterizing these particular young dogs.

Further experiments with rats likewise gave some data at variance with others. Consideration of the 'degree of immaturity' of the animals yielding the respective data, in these cases known by the age and body weights of the rats, again suggested very definitely that there is a factor related in some way to the effect of lactose on calcium and phosphorus utilization. In reporting these experiments, therefore, we have taken this possible relationship as the theme of this paper.

PART I

BALANCE STUDIES IN DOGS

Plan of experiment

The plan of investigation followed in part I of this study consisted of the determination of calcium and phosphorus balances in consecutive periods differing in only one respect, namely, the presence or absence of lactose in the diet. In comparable experimental periods diets were used that were low in either calcium or phosphorus. Other factors taken into account were the possible influence of the protein in the diet and the age of the dog. Either dried coagulated egg albumen or the phospho-protein casein were used as the source of protein in comparable periods. One mature dog and two immature animals yielded the data reported in part I of this paper.

In the experimental routine the dogs were fed the particular diet in question for a period of 5 days to allow some adjustment to the new dietary conditions; this was followed by a 5-day period during which collections were made and the balances determined. At the beginning and the end of each period the dogs were fed carmine to mark the stools and catheterized to secure complete emptying of the urinary bladder. The dog's food for each period was weighed out at the beginning and a sample taken at that time for chemical analysis. The urine was collected daily, measured and made up into a composite sample which was analyzed. The treatment of the feces was similar except that the composite sample was dried, weighed, ground and saved for analysis in a sealed bottle. Blood analyses for calcium and phosphorus were performed in all experimental periods.

Methods of analysis

For the calcium analyses of food, feces and urine, McCrudden's ('10, '11) method was used. Blood calcium was determined by the Clark-Collip ('25) modification of the Kramer-Tisdall method. Neumann's ('02) technic was employed for the phosphorus analyses performed on the food, feces and urine, and the blood plasma phosphorus was determined by Briggs' ('24) modification of the Bell and Doisy procedure.

Composition of diets

The compositions of the diets and salt mixtures used in this study are given in tables 1 and 2. In constructing the experimental rations the kilo-unit plan described by Cowgill ('23) was followed.

The duration of each balance period was 5 days with a 5-day interval between them to allow an adjustment to the new dietary regime. The plan of experiment involves the assumption that the utilization of calcium and phosphorus over a period of 15 days is constant except for the reaction of the animal toward the one change made in the diet, namely, the substitution of lactose for some of the sucrose.

RESULTS

Adequacy of technic. Bauer, Albright and Aub ('29) showed that it is possible under uniform conditions to obtain duplicate negative calcium balances on human beings. On the same person consecutive balance experiments checked satisfactorily but there were considerable variations between different subjects on the same diet that were not readily explained. Our first experiment was designed to yield confirmatory data on this point and to assure us that our technic was adequate for the problem being studied. The result was most satisfactory and readily confirmed in later experiments. As an example of these data we present the results for dog 2, period 1, shown in table 3. It will be noticed that two separate experiments with the diet of 'period 1' were made with dog 2. The results with respect to the calcium balances are almost identical; the data pertaining to phosphorus do not agree so well. In explanation of this difference we can only offer the consideration that phosphorus seems to play a more varied role in metabolism and therefore may not be as readily controlled as calcium.

Effect of lactose during a low-calcium dietary regime. In all of the experiments the diets were made up with the carbohydrate constituting 40% of the mixture. When lactose was added it was substituted for one-half of the sucrose. In

TABLE 1

Composition of diets

Ration 1: For low-calcium regime

INGREDIENT	GRAMS	CALORIES	PER CENT
Egg albumen (coagulated), 11.91% N	5.78	17.2	32.0
Lard	2.80	25.2	15.5
Butter fat	1.20	10.8	6.6
Sucrose	7.25	29.0	40.2
Cellu flour	0.6		3.3
Salt mixture no. 1	0.43		2.4
Total—kilo unit	18.06	82.2	100.0

Ration 2: low Ca plus lactose; same as ration 1 except that lactose is substituted for one-half the sucrose.

Ration 3: low P; same as ration 1 except that salt mixture no. 2 is used in place of salt mixture no. 1.

Ration 4: low P plus lactose; same as ration 2 except that salt mixture no. 2 is used in place of salt mixture no. 1.

Ration 5: low Ca; same as ration 1 except that 5.46 gm. of casein containing 12.6% N replaces the egg albumen.

Ration 6: low Ca plus lactose; same as ration 5 except that lactose is substituted for one-half the sucrose.

TABLE 2

Composition of salt mixtures

COMPONENTS	OSBORNE-MENDEL ('19)	NO. 1	NO. 2
	gm.	*gm.*	*gm.*
Calcium carbonate	134.8	....	134.8
Magnesium carbonate	24.2	24.2	24.2
Sodium carbonate	34.2	34.2	34.2
Potassium carbonate	141.3	141.3	141.3
Potassium iodide	0.02	0.02	0.02
Phosphoric acid	103.2	103.2	....
Hydrochloric acid	53.4	53.4	53.4
Manganese sulfate	0.079	0.079	0.079
Sulfuric acid	9.2	9.2	9.2
Citric acid	111.1	....	....
Ferric citrate	6.34	6.34	6.34
Sodium fluoride	0.248	0.248	0.248
Potassium alum	0.0245	0.0245	0.0245
Total	618.1115	372.2115	403.8115
Amounts used in grams:			
Per 100 gm. of food	4	2.41	2.61
Per kilo unit	0.74	0.43	0.47

TABLE 3

Calcium and phosphorus balances in dogs on diets with and without lactose

BASIC DIETARY REGIME	DOG NO.	DIET NO.[1] (L = LACTOSE)	CALCIUM PER DAY (AVERAGE)				PHOSPHORUS PER DAY (AVERAGE)				BLOOD (PER 100 CC.)	
			Intake	Output		Balance	Intake	Output		Balance	Ca	P
				Feces	Urine			Feces	Urine			
			gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	mg.	mg.
Low calcium	1	5: without L	0.101	0.170	0.008	—0.077	1.119	0.158	0.974	—0.013	12.1	5.9
	Immature	6: with L	0.121	0.110	0.017	—0.006	1.113	0.126	0.850	+0.137	12.8	5.9
	2	1: without L	0.061	0.161	0.026	—0.126	0.540	0.084	0.517	—0.061	12.1	5.6
	Immature	1: without L	0.066	0.168	0.026	—0.128	0.547	0.090	0.498	—0.041	11.6	6.7
		2: with L	0.061	0.109	0.010	—0.058	0.556	0.065	0.480	+0.011	11.8	4.7
	2	5: without L	0.117	0.095	0.004	+0.018	1.291	0.083	1.127	+0.081	12.2	5.7
	Immature	6: with L	0.141	0.038	0.015	+0.088	1.285	0.088	1.033	+0.164	12.3	6.1
	3	5: without L	0.069	0.063	0.004	+0.002	0.791	0.240	0.536	+0.015	10.8	4.7
	Mature	6: with L	0.082	0.079	0.003	0.000	0.788	0.457	0.322	+0.009	11.1	4.8
Low phosphorus	1	3: without L	0.565	0.586	0.019	—0.040	0.278	0.134	0.194	—0.050	12.0	4.8
	Immature	4: with L	0.622	0.394 [2]		+0.228	0.268	0.190 [2]		+0.078	12.3	6.0
	2	3: without L	0.639	0.509	0.010	+0.120	0.287	0.087	0.261	—0.061	12.1	6.3
	Immature	4: with L	0.705	0.520 [2]		+0.185	0.276	0.083 [2]		+0.193	13.6	6.1
	3	3: without L	0.400	0.405	0.006	—0.011	0.153	0.103	0.061	—0.011	10.6	4.8
	Mature	4: with L	0.441	0.459 [2]		—0.018	0.146	0.164 [2]		—0.018	11.7	4.0

[1] For detailed composition of these diets see table 1.

[2] Total for feces and urine; could not collect these excreta separately. See text for discussion of laxative effect of diet 4.

Inouye's ('24) study of the value of lactose in preventing and curing parathyroid tetany, the ratio of the amount of casein in the diet to the lactose fed was found to be very important. Various diets which failed to prevent tetany had casein/lactose values as high as 8; rations which were borderline had values ranging from 1 to 4; diets which definitely protected against the development of tetany had values as low as from 0.08 to 1. In view of these data it was our expectation that any influence of lactose on calcium and phosphorus balances would be most marked when the ratio of protein to lactose in the experimental diets approximated the value of 1. Actually the ratio for our rations proved to be 1.3. A summary of the data is presented in table 3.

Examination of table 3 reveals that in the case of the two immature dogs on a low-calcium regime, substitution of lactose for one-half of the sucrose resulted in marked improvement in both the calcium and the phosphorus balances. In the case of dog 1, the use of lactose was associated with a change of the calcium balance from a daily loss of 0.077 gm. to approximate equilibrium and the phosphorus balance was altered from a moderately negative condition to one distinctly positive. Dog 2 was used in two separate experiments, one with diets 1 and 2, in which egg albumen was the protein used, and the other with diets 5 and 6 which contained casein as the protein; in the latter case the balances without lactose were positive, and the addition of this carbohydrate was associated with increased retention of both calcium and phosphorus. In contrast to these results obtained with immature dogs one sees the data for the mature animal, dog 3, indicating insignificant changes in the calcium and phosphorus balances, the larger difference in the case of the phosphorus being within the range of differences noted in duplicate experiments (dog 2, period 1).

The changes in the utilization of calcium associated with the presence of lactose are large enough to suggest that this carbohydrate acts in some way to diminish the rate of excretion of calcium. The increase in the amount of Ca utilized by dog 2, when changed from diet 1 to diet 2, is slightly greater than the total quantity of the element furnished in the ration.

Effect of lactose during a low-phosphorus dietary regime. For this phase of the investigation rations 3 and 4, low in phosphorus content, were devised (table 1), the salt mixtures being altered to meet the requirements of the experiment. Examination of the data in table 3 reveals that in immature dogs the use of lactose together with a dietary low in content of phosphorus results in improvement of both the calcium and the phosphorus balances. This result is again in marked contrast to that characterizing the mature animal.

Egg albumen was used as one of the proteins of the diets because the plan of experiment required use of a diet low in phosphorus content. Being available in quantity, and lacking both calcium and phosphorus, the two variables of greatest interest in these experiments, this protein was selected.

Bateman ('16) has shown that ingestion of appreciable quantities of raw egg-white results in diarrhea in both animals and man. For our experiments, therefore, the egg albumen (commercial scales) was coagulated by heat, dried and ground before being incorporated in the test diets. When the egg albumen rations were fed, the resulting freshly voided feces had a greenish color which rapidly turned black on standing; this necessitated the use of carmine rather than charcoal to differentiate the stools.

It is an interesting fact that the favorable influence of lactose on the utilization of calcium and phosphorus was observed whether the protein was coagulated egg albumen or the phospho-protein casein.

Lactose had a definitely laxative effect in all the dogs. Egg albumen acted similarly. In the experiments with diet 4 (low phosphorus plus lactose) the cumulative effect of lactose and the protein on the consistency of the feces was such that a separation from the urine could not be made by the method used. Under these conditions, nevertheless, the favorable influence of lactose on the utilization of calcium and phosphorus was observed, from which it appears that even when considerable laxation prevails lactose still has the property of increasing the availability of calcium and phosphorus to the immature animal.

The fact that no such effect was observed in our mature dog suggests that the age of the animal, or its degree of maturity, is significant in this connection. It is possible that the age factor is significant because in the young animal there is a more active bone metabolism associated with rapid growth, whereas in the mature organism there is no necessity for utilization of calcium and phosphorus beyond the requirements for maintenance except under particular conditions such as pregnancy and great activity of the mammary glands during lactation. It appears that the production of cataract in rats by high-lactose diets is likewise related in some way to the age of the animal, young rats being definitely more susceptible in this respect (Mitchell and Dodge, '35).

PART II

EXPERIMENTS WITH RATS

Does lactose act to favor absorption or to diminish the excretion of calcium and phosphorus? Examination of the data in part I for distribution of excreted calcium and phosphorus between the feces and urine does not reveal a definite answer to this question. Bergeim ('26) has shown that in both normal and rachitic rats there is good absorption of calcium from the small intestine, the secretion of phosphate into the gut favoring such absorption. Bergeim's method consists essentially in adding to the ration a substance which is practically unabsorbable, ferric oxide, for example, and then studying the ratio of this substance to calcium and to phosphorus in different parts of the gastro-intestinal tract. In this connection it is pertinent to point out that the experiments, in which Bergeim demonstrated the favorable effect of lactose on the retention of calcium and phosphorus, were conducted on young rats. Using Bergeim's method and a diet low in calcium content one might expect the absorption of calcium in the upper part of the intestinal tract to be fairly complete. If this assumption is correct, then differences in increases of calcium concentration in lower parts of the tract should be due to changes in rates of elimination. It was therefore decided to

test this idea on rats using Bergeim's technic. These experiments are described in this section. In the course of this phase of the study additional evidence was obtained indicating the importance of degree of maturity of the organism in determining the effect of lactose on the utilization of calcium and phosphorus.

Analytical methods

The methods for determination of calcium and phosphorus were the same as those employed in the experiments described in part I. Phosphorus was determined colorimetrically because the quantity of iron in the samples was great enough to interfere seriously with the usual molybdate procedure. In the first experiments described below iron was determined by the simple method recommended by Bergeim ('26), but owing to the variable results obtained, the colorimetric thiocyanate technic as improved by Marriott and Wolf ('05–'06) was substituted and found to yield satisfactory duplicate results.

Preliminary experiments with a low-calcium diet

These trials of the Bergeim technic were performed on ten female rats distributed between two groups, one receiving diet 5 (low-calcium) and the other group diet 6, which was the same as diet 5 except that lactose was present to the extent of 20% replacing an equivalent amount of sucrose. Failure to secure any clear-cut results in these experiments led to a search for an explanation. To eliminate the possibility that the Bergeim method for determination of iron was inadequate under our conditions of study, the colorimetric thiocyanate method as improved by Marriott and Wolf ('05–'06) was substituted for the remaining experiments. There also occurred to us the possibility that significant but unappreciated quantities of calcium were being poured into the gastro-intestinal tract sufficient to off-set any effect that feeding a low-calcium diet might have. Therefore data on the amounts of calcium present in various secretions of the alimentary tract were sought in an attempt to weigh this possibility. From the data

reported by Austin and Mathews ('27) and Walch and Ivy ('27–'28) it appears that the concentrations of calcium in the saliva, gastric juice, pancreatic juice, bile and secretions of the lower ileum of the dog approximate those found in the blood, ranging from as low as 6 mg. per 100 cc. for gastric juice to as high as 11 for gall bladder bile. Obviously, when the amount of calcium contained in the food is very low, the quantities of this element contributed by the alimentary secretions assume great importance. Therefore we are justified as assuming as correct the view that the advantages one might expect to gain in using a low-calcium diet in this type of experiment are nullified by the appreciable amounts of calcium poured into the gastro-intestinal tract in the course of normal digestive processes. For this reason we did not repeat this phase of the study.

Experiments with complete diets containing varying amounts of lactose

Six groups of rats, two females in each, were used for this phase of the study. The animals were classified by weight and then fed complete diets containing levels of lactose ranging from 0 to 60%. Six rations were thus tested. The feeding was continued for a week, and then a 3-day collection of feces was made, specimens soiled with urine being discarded. The feces were dried, ground and analyzed for iron, calcium and phosphorus, as was the food. The ratios of iron to calcium and to phosphorus of both the food and the feces were then computed and compared. The results are presented graphically in figure 1.

In interpreting figure 1, it should be borne in mind that the differences between the heights of the columns in any given group are the significant features to be studied. In the upper row of columns are presented the ratios of calcium/iron and phosphorus/iron resulting from the analyses of the diets used. The middle row gives the calcium/iron ratios yielded by each of the two animals on the experiment; in the lower row are shown similar data referring to the phosphorus/iron ratio.

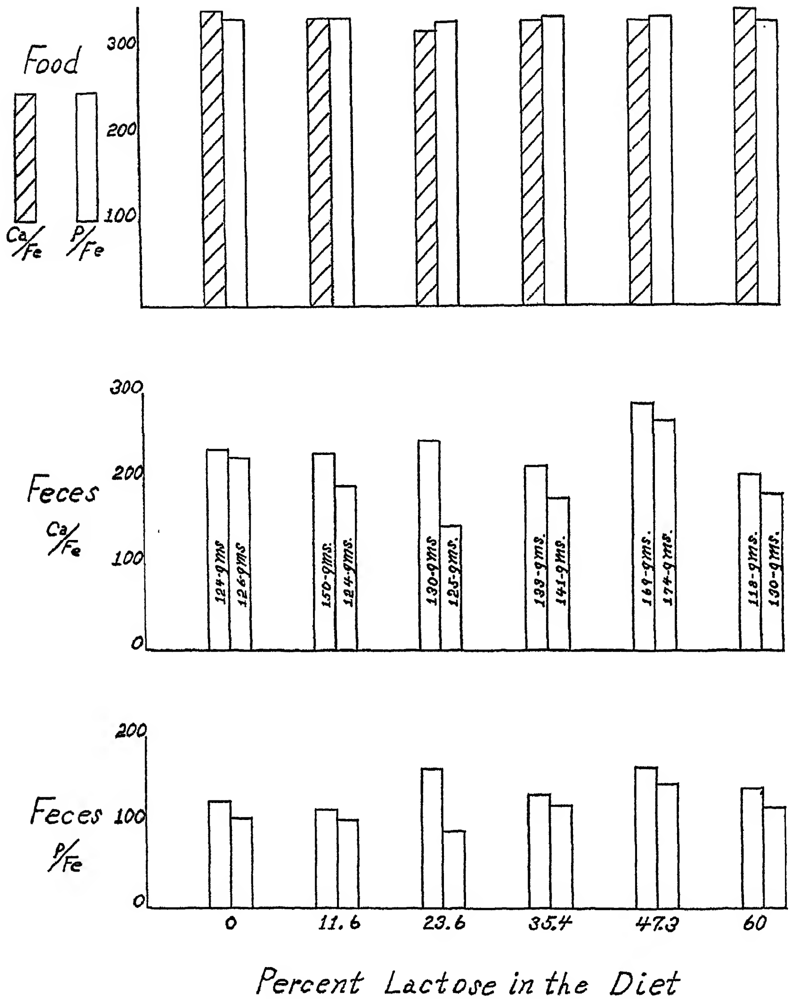

Fig. 1 The effect of varying amounts of lactose in the diet of rats on the ratios of calcium and phosphorus to iron in the feces studied by the Bergeim ('26) technic. The results presented in the two lower rows of columns—data pertaining to the feces—were obtained with two female rats for each experimental diet. Then body weights of the animals are given in the respective columns of the middle row. It will be noticed that the columns in the middle row, Ca/Fe, for the diet containing 47.3% of lactose are the highest, indicating the smallest utilization of calcium; these particular rats were the heaviest of the entire group and, by reference to figure 2, had passed the period of most rapid rate of growth.

The variations observed in the columns of the two lower rows are worthy of comment. In arriving at the ratio values for the diets, it is apparent that the additive errors of two chemical determinations are involved; furthermore, the determinations represented by the upper row of columns were made on six different rations. The fact that only very slight differences are shown in the columns of the upper row therefore indicates that the cause of variation in results summarized in the two lower rows of figure 1 must be sought elsewhere than in error of chemical analyses. Evidently they are due to biological factors difficult to control; that appreciable variability of results is common in work of this type appears evident also from the data of Bauer and associates ('29) who made very careful balance studies on human subjects and yet, in many instances, observed variations that could not be explained.

Examination of the columns in the middle row reveals that the two animals receiving the diet with 47.3% lactose showed the least utilization of calcium of all the groups, and the degree of utilization in this case was quite small. These two animals were the heaviest of the entire group, weighing 169 and 174 gm., respectively. It will be recalled that in the experiments on dogs, described in part I of this paper, the mature animal failed to show any increase in utilization of calcium and phosphorus when lactose was fed. We therefore considered the possibility that the degree of maturity of the rats used in these experiments was playing a role.

All of the rats were females obtained from four different litters; in age they ranged from 67 to 71 days at the time the experiments were begun. The weights of the animals, corresponding to the time when the data yielding the ratios indicated in figure 1 were obtained, ranged from 118 to 174 gm. These weights are indicated by the figures on the respective columns. It will be noticed that the two rats which showed the least utilization of calcium weighed the most, namely, 169 and 174 gm.

The question then presented itself: When may a female rat be said to have reached maturity or, as far as the problem of the moment was concerned, at what level of body weight (or age) is there a marked reduction in rate of growth, and with this a corresponding decrease in the demands of the body for a substance like calcium?

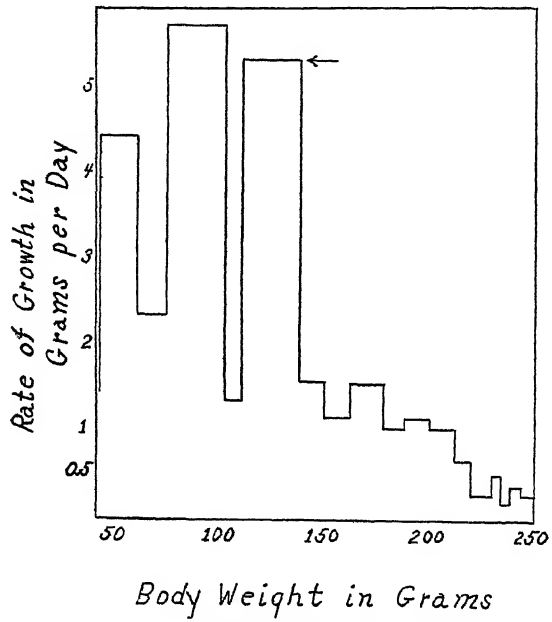

Fig. 2 This plot, based on the data of Smith and Bing ('28) for female rats, indicates that when a body weight of about 137 gm. has been attained, there is a marked decrease in the rate of growth referred to body weight. The variations shown on the graph previous to the arrow are not readily explained.

Smith and Bing ('28) have published data on the growth of female rats in this colony. Using their data we have plotted average daily gain in weight against average body weight for the same period. The result of such a plot is shown in figure 2.

From figure 2 it appears that when a female rat of this colony has attained the weight of about 137 gm. (see arrow), an average figure having a standard deviation of 12.8 and a coefficient of variation of 9.3%, that rat may be said to have

passed the period of most rapid growth, and therefore 'matured' in this sense. It is obvious that such a result as we have just cited may very well be conditioned by numerous factors operating in the colony of this laboratory.

The two animals that were definitely larger than the limit suggested by figure 2 weighed 169 and 174 gm., and they were the only rats used in this experiment whose data failed to confirm to those obtained in the experiments with immature dogs described in part I. Assuming the correctness of the thesis suggested by the dog experiments and its applicability to the rat, we might expect that in these two rats lactose would not have a favorable influence on calcium utilization. Added confirmation of this idea was obtained by reexamination of the absorption data obtained in the preliminary series of rat experiments, namely, those in which a low-calcium diet and Bergeim's iron method were used. The weights of the four rats in this series, which had given data not in harmony with those yielded by the other animals, were found to range from 170 to 210 gm. On the other hand, the remaining six rats of the first series weighed from 90 to 140 gm., and gave data indicating a favorable effect of lactose on calcium utilization.

Although we realize that other factors may operate to cause the observed variations in our results, we believe our data indicate that a certain degree of immaturity of the animal must play a role. If we are correct in this belief, then it appears that extended comparative studies on the same rat, where it is intended that the animal serve as its own control, will not yield the same results at all times. This factor, which for the present we shall call 'degree of immaturity,' without attempting to define it more explicitly, may assist in explaining the contradictory results reported in the literature.

SUMMARY AND CONCLUSIONS

The possible influence of the presence of lactose in the diet on the utilization of calcium and phosphorus was studied by performing balance experiments in two young dogs and one mature animal. The ratios and the amounts of these two elements were carefully controlled. Likewise the effect of the

phospho-protein casein was compared with that of egg albumen.

The data obtained from the young animals indicated very definitely that the presence of lactose in the diet favorably influences the utilization of calcium and phosphorus; no such result was obtained with the mature dog, however.

Attempts were made by experiments on rats, using Bergeim's technique, to determine whether this favorable action of lactose is due to increased absorption from or diminished excretion into the intestine. It appears that the use of a low-calcium diet in tracing the absorption of this element is of practically no value because of the quantities of lime thrown into the alimentary tract by the digestive secretions. The data obtained from rats showed too great variation to be judged as entirely satisfactory. Nevertheless, they very definitely confirmed the dog experiments indicating that 'degree of immaturity' plays a role in determining the effect of lactose on the utilization of calcium by a given organism. These experiments on rats support, but do not prove, the view that lactose acts to diminish the excretion of calcium into the intestine.

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THE TOXICITY OF HIGH-GLIADIN DIETS. STUDIES ON THE DOG AND ON THE RAT [1]

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In the course of an investigation of the influence of diet upon the regeneration of serum protein in the dog (Melnick, Cowgill and Burack, '36), it was essential to determine previously for each of the tested proteins the minimal quantity required to secure nitrogen equilibrium. The source of the proteins and vitamin adjuvants, the construction of the diets and the nitrogen balance studies, conducted with lactalbumin, serum protein, casein or gliadin as the sole protein in the rations, are described elsewhere (Melnick and Cowgill, '37). When gliadin is fed as the sole source of dietary protein, there is a deficiency with respect to the essential amino acid, lysine. Probably it is for this reason that we found it necessary to feed the protein at a very high level of intake before nitrogen equilibrium was attained. Indeed, fully three times as much gliadin as compared with lactalbumin was essential for satisfying the general nitrogen requirements of the organism (Melnick and Cowgill, '37). In the course of these experiments certain unexpected toxic reactions were noted in the dogs subsisting on the high-gliadin diets. Obviously, the phenomena were not due to the amino acid deficiency characteristic of the protein, for it was with the high-gliadin diets alone that the reactions were observed. Only when the protein was consumed in amounts approaching and surpassing the minimal

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[2] Alexander Brown Coxe Fellow, 1936–1937.

value for nitrogen equilibrium were these manifest. Is it possible that the ingestion of a foodstuff, either in the form of protein or its constituent amino acids, at a level of intake satisfactory for the general nitrogen requirements of the organism can be toxic? The important implications of such a possibility warrant careful study.

The phenomena. On the seventh to the ninth day of the dietary regime the dogs fed a high-gliadin diet exhibited a peculiar behavior. The initial reaction may be characterized by the term 'running fits.' The dog suddenly begins to act in a strange manner and apparently is greatly frightened. He looks around and begins barking as if in severe pain. The animal then runs wildly, hurling itself blindly against the walls of the cage. This occurs generally for from 2 to 10 minutes. Subsequently, during the recovery period, the animal appears bewildered but otherwise normal. In some cases one attack may be followed immediately by another.

The more intense reaction which comes on with prolonged feeding of the high-gliadin diet bears a striking resemblance to epileptic convulsions. The animal first goes through the initial stage described above. This is followed by intense foaming at the mouth, the animal running around wildly attempting to 'catch its breath.' Suddenly the head is pulled to one side and, like the rest of the body, is held stiffly. The animal drops heavily on its side with all the muscles of the body undergoing rapid, fixed, rhythmic contractions. The dog then hurls itself furiously about the cage. With the return of normal respiration the animal lies relaxed and appears to be in a stupor. Several minutes later the animal is on its feet, apparently normal. Usually the frothy saliva is colored with blood due to the tongue or cheeks having been bitten. The bladder and the bowels are frequently emptied during these convulsions. We have noted several such seizures in the same animal following each other in rapid succession. At first, the toxic reactions were observed just after the gliadin diets were offered to the animals or during the time the rations were being ingested. Subsequently, convulsions were noted to occur

irrespective of the feeding schedule and, therefore, were interpreted as being neither conditioned nor due to the presence of a recently ingested meal in the stomach. The fact that these attacks are related in some way to the protein used in the diet is obvious from table 1.

The phenomena are not due to intestinal parasites. Dog no. 9 never showed these reactions, since the gliadin was only fed at that level constituting 13% of the caloric intake. Dog no. 10 was fed the gliadin diet at 7.45, 10.6 and 22% levels consecutively, developing convulsions only with the last-named

TABLE 1

Toxic effects of feeding high-gliadin diets

DOG NO.	BREED (SEX)	WEIGHT[1]	PER CENT OF CALORIES IN THE FORM OF GLIADIN					
			7.45	10.6	13.0	16.0	22.0	25.0
		kg.						
9	Spitz (female)	8.00	----	----	N.R.	----	----	----
10	Fox-terrier (female)	8.95	N.R.	N.R.	----	----	R.F. and E.C.	----
11	Bulldog (female)	7.76	----	N.R.	----	R.F. and E.C.	----	R.F. and E.C.
13	Mongrel (female)	9.23	----	----	N.R.	----	----	R.F.

[1] These values are calculated for the animals when adjusted to an optimal nutritive condition, as estimated by the nutritive index formula (Cowgill, '28).

----, indicates gliadin not fed at that caloric level.
N.R., indicates no reaction.
R.F., indicates 'running fits.'
E.C., indicates epileptiform convulsions.

ration. Dog no. 11 was fed the 10.6 and 16.0% diets consecutively with convulsions occurring only with the latter diet. Inasmuch as worms, especially tape- and hook-worms (Hutyra and Marek, '26; Hall, '25) have been reported to cause convulsions in dogs, attempts were made to determine whether intestinal parasites constituted the etiologic agent. Dogs nos. 10, 11 and 13 were given vermifuge, arecoline [3] being employed for removing the tape-worms and tetrachlorethylene [3] for the

[3] Obtained from the Parke, Davis and Co., Detroit, Mich.

round-worms. Dog no. 10 was then fed a stock diet consisting of a mixture of natural foods and never did develop any untoward reactions. Dogs nos. 11 and 13 were fed gliadin at the 25.0% level and were observed to show the characteristic symptoms after the eighth day on this diet. The feces of these dogs were free of tape-worm eggs and contained a small number of hook-worm ova.[4] Experimentation with these animals was discontinued. It was surprising to note that fully a week was required before these dogs, subsisting on the stock diet, ceased showing an occasional convulsion.

These seizures are not due merely to a high-protein diet per se. This is obvious, when it is considered that the casein diet (Cowgill, '28), commonly used in this laboratory, which contains protein to the extent of approximately 28% of the caloric intake, has never caused similar reactions in dogs, including our own experimental animals even when subsisting on the diet for as long as 18 months.

The toxic reactions are not due to a significant uremia. The behavior of the animals subjected to the high-gliadin diets suggested that there might be an accumulation of some toxic substance, and that the disappearance of the reaction occurred only when this substance was reduced below a threshold value. Gliadin is composed of as much as 43.7% glutamic acid (Mendel, '23). This amino acid, when introduced parenterally, has been shown to be toxic for rabbits (Johnston and Lewis, '28). In the case of man, Lewis and co-workers ('18) have reported that glutamic acid ingestion is productive of nausea, general malaise and oliguria. Our dogs all showed an associated oliguria, the urine excretions falling to from one-third to one-half of their normal volumes. This finding is all the more significant in view of the fact that the dogs subsisted on a high-protein diet and such diets, by giving rise to nitrogenous end-products, tend to exert a diuretic effect. MacKay ('33) has noted that the administration of glutamic acid to

[4] The writers acknowledge their indebtedness to Dr. Telford W. Workman for his assistance in investigating the possibility of intestinal parasites being responsible for the appearance of the seizures noted.

rats which have been maintained upon a basal diet containing casein, results in renal hypertrophy; the maximum effect was observed in the relatively short period of 10 days. Indeed, certain amino acids have been reported (Kotake, '34) to cause nephrosis, when injected intraperitoneally. Glutamic acid and cystine appeared to compensate each other since injections of both resulted in no nephrosis. Contrary to these suggestive findings, the relative non-toxicity of glutamic acid as contrasted with that of tyrosine and cystine has been reported (Sullivan, Hess and Sebrell, '32; Lillie, '32). Thus, the toxicity of the gliadin, observed in the present study, may perhaps be due to glutamic acid. However, in view of our own rat experiments and the additional tests on the dog, cited below, this does not seem to be an adequate explanation.

Seeking confirmatory evidence, three additional dogs were fed the 22% gliadin diet at the usual level of intake of 70 calories per kilogram of body weight together with the vitamin adjuvants. Determinations were made of the blood non-protein nitrogen (Folin and Wu, '19) before and after the use of this diet. The results of this study indicated that the convulsions could not be attributed to a significant uremia. The non-protein nitrogen values before and after the administration of the gliadin ration were essentially normal. In all cases the reactions subsequently disappeared when the animals were fed stock diets with no vermifuge treatments.

Experiments with the rat. Numerous investigators have fed rats artificial 'synthetic' diets in which gliadin constituted practically the sole source of protein. No convulsive reactions were ever reported. This may be due to a species difference or due to the fact that the ingested gliadin constituted only 11 to 13% of the caloric intake. In the present study attempts have been made to determine whether high-gliadin diets are toxic for rats.

The possibility that the substance, active in producing the seizures noted in our dogs, may be merely a contaminant of the gliadin preparation was also studied. The investigations of Franke ('34) of a toxicant occurring naturally in certain

samples of plant foods have indicated that this substance is carried by the protein fraction. Subsequent studies (Franke and Painter, '36) with the gluten protein from toxic wheat have shown that selenium in organic combination with the protein is responsible for the toxicity of the foodstuff. The gliadin employed in our investigations was prepared from gluten flour [5] (Nolan and Vickery, '36). In view of these facts, might it not be possible that selenium may be present as a contaminant of the gliadin and thus be the toxic substance in the protein preparation? However, analyses [6] of the gliadin indicated that our preparation was free from selenium. With respect to other possible inorganic contaminants of the protein, it is pertinent to point out that the preparation contained only 0.15% ash. Furthermore, the gliadin was isolated by means of a 75% alcoholic extraction of the gluten flour, a procedure which tends to leave the inorganic salts behind with the insoluble residue.

The possibility of some other toxic substance adhering to the gliadin preparation was investigated in the rat, using growth data as the criteria. Thirty-three male rats, weighing within 40 to 50 gm. at weaning, were employed. These animals were weaned at 21 days of age and distributed into six groups. In no case were two rats from the same litter placed in the same group. The animals were fed artificial 'synthetic' diets containing casein,[7] gliadin, or gliadin plus lysine as the sole source of protein. The proteins were fed at two levels, '18%' and '36%' (by weight) of the dietary mixture. The casein contained 13.37% nitrogen; the gliadin, 14.81%. These analyses indicate that both proteins were 84.1% pure (Jones, '31). Thus, the percentages of pure protein in the dietary mixture were actually 15.14 and 30.28%, and constituted 12.4 and 25.6% of the caloric intake, respectively. For the sake of

[5] Obtained from the Battle Creek Food Co., Battle Creek, Mich. It is reported as 'containing 85% gluten.'

[6] We are indebted to Dr. Alvin L. Moxon for these analyses. These determinations were carried out in Franke's laboratory, South Dakota State College of Agriculture and Mechanic Arts, Brookings, S. D.

[7] Obtained from the Lister Bros., New York, N. Y.

convenience, the diets will be referred to as the 18 and 36% protein rations. One of the 18% gliadin diets was supplemented with *dl*-lysine dihydrochloride [8] to the extent of 1.62% of the dietary mixture. Osborne and Mendel ('14) have shown that 'normal' growth in the rat may be promoted when a maintenance ration containing gliadin as the sole protein is supplemented with lysine. In their work gliadin was fed at an 18% level by weight and lysine added equivalent to 3% of the gliadin, i.e., 0.54% of the total ration. Since only one-third of *dl*-lysine dihydrochloride (Berg, '36) is physiologically active with respect to promoting growth, we have supplemented the gliadin diets with three times that quantity, namely, 1.62% of the ration.

In order to compare favorably the experiments with the rat with those conducted on the dog, the diets in both studies were constructed on the kilo-unit (Cowgill, '23; Melnick and Cowgill, '37). The vitamin adjuvants were the same but the quantities given to the rat were different from those fed the dog. The daily administration to the rat of 200 mg. of a rice polishing extract [9] and 200 mg. of liver extract no. 343 [10] satisfied the vitamin B (B_1) and G (B_2) requirements, respectively. For vitamins A and D, 20 mg. of a cod liver oil concentrate [11] were administered every 4 days. These quantities of the vitamin adjuvants have been found to supply abundantly the needs of the growing and adult rat.[12]

From the time of weaning all the rats were fed the 18% casein diet plus the vitamin supplements in order to allow the animals adequate time for adjustment to a new dietary regime. After each rat had consumed 250 calories of this ration, it was fed 650 calories of one of the six diets employed in this study. The animals were weighed daily. At the end of the experimental period the rats were killed. The kidneys were

[8] Obtained from the Eastman Kodak Co., Rochester, N. Y.

[9] 'Ryzamin,' obtained from the Burroughs Wellcome Co., Tuckahoe, N. Y.

[10] We are indebted to the Eli Lilly Co., Indianapolis, Ind., for a generous supply of this material in powdered form.

[11] Kindly furnished by the Health Products Corp., Newark, N. J.

[12] Personal communication from Aline U. Orten.

removed and weighed; the average weights were correlated with surface area, estimated from the average body weight values (Carman and Mitchell, '26). Table 2 summarizes the experimental findings.

A consideration of the data presented in the table indicates that the protein, isolated from wheat gluten and used in the present study, was a good gliadin preparation as judged by

TABLE 2

Summary of the data obtained with rats subsisting on artificial 'synthetic' diets containing casein, gliadin or gliadin plus lysine as the sole source of protein

GROUP (NUMBER OF RATS)	DIET	AVERAGE FOOD INTAKE PER DAY	AVERAGE BODY WEIGHT			KIDNEY WEIGHTS [1]		
			Initial	End of period I	End of period II	Range	Average	Per 100 sq.cm. of surface area
		gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
I	18% casein	6.3	44	71				
(8)	18% casein	8.6			126	0.411–0.549	0.464	0.162
II	18% casein	6.1	44	71				
(4)	18% gliadin	5.4			70	0.306–0.343	0.331	0.171
III	18% casein	6.1	45	73				
(4)	18% gliadin + 1.62% *dl*-lysine · 2 HCl	7.1			101	0.382–0.413	0.401	0.162
IV	18% casein	6.3	46	74				
(7)	36% casein	9.0			156	0.566–0.743	0.632	0.192
V	18% casein	6.3	45	75				
(5)	36% gliadin	5.7			79	0.379–0.460	0.422	0.201
VI	18% casein	6.3	46	75				
(5)	36% gliadin + 1.62% *dl*-lysine · 2 HCl	7.8			129	0.513–0.559	0.542	0.186

[1] The average weight of the two kidneys of each rat were used in calculating these values.

the available criteria. It was not contaminated to any appreciable extent by any other proteins found in wheat gluten. This is evident when comparisons are made of the rate of growth of rats subsisting on the 18% gliadin diet with that noted with the control animals fed the 18% casein diet. The ingestion of the gliadin ration supplemented with lysine promoted growth at the rate of 1.5 gm. per day. No obvious toxic

symptoms were noted in these rats. These were not expected since the gliadin constituted only 12.4% of the caloric intake.

Rats fed a 36% gliadin diet were stunted almost to the same extent as those subsisting on the 18% ration. This finding is significant because dogs subsisting on a 36% gliadin diet (25.6% of the caloric intake) will attain nitrogen equilibrium (Melnick and Cowgill, '37). Gliadin does contain 0.63% of lysine (Mendel, '23), but this is not sufficient to permit growth in rats fed the usual 18% protein diet. However, with respect to the attainment of nitrogen equilibrium in adult animals, it appears that sufficient lysine was ingested, when the dogs consumed the high-gliadin diet, associated with the positive balances recorded (Melnick and Cowgill, '37). The possibility existed, therefore, that rats fed a 36% gliadin diet might exhibit appreciable growth. The experimental findings proved that such is not the case. Could it be possible that the inhibition of growth was due not to the lysine deficiency but to the toxic principle associated with the gliadin preparation? Supplementation of the gliadin ration with lysine resulted in a normal rate of growth, 3.1 gm. per day. The amount of lysine added to the 36% gliadin ration was identical with that supplementing the 18% diet, but the growth response in rats ingesting the former diet was actually two times greater. Apparently the lysine content of gliadin, although insufficient in itself to permit growth in rats fed the 36% protein diet becomes significant when combined with the lysine supplement: it is nutritionally significant. This finding appears to confirm our suggestion that the attainment of nitrogen equilibrium by the dog subsisting on the high-gliadin diet is due to the ingestion of sufficient lysine as part of the protein intake. No obvious 'toxic' symptoms were observed with the rats subsisting on the high-gliadin diet other than inhibition of growth, and this was corrected by adding lysine to the diet.

The possibility that sufficient glutamic acid may be ingested as part of the protein intake to cause a nephrosis in rats subsisting on our gliadin diets was also studied. The kidney weights (table 2) indicate no significant atrophy or hypertrophy of that organ. This is especially true if these values are

correlated with the surface areas of the animals. The slightly larger kidney weights recorded for the animals subsisting on the 36% protein rations are in accordance with the finding that the ingestion of high-protein diets by the rat results in renal hypertrophy (Osborne, Mendel, Park and Winternitz, '27). Histological study[13] indicated that the kidneys of rats fed the 36% gliadin diet were normal. Furthermore, the urines obtained from these animals were free from protein.

The toxicity of the high-gliadin diets may be due to a protein sensitization.[14] Subsequent to the protein minima studies conducted with dogs nos. 10, 11 and 13, the animals were removed from the metabolism cages and returned to the dog house. Approximately 15 months later, dog no. 10 was brought back to the laboratory to be used as the experimental subject in another investigation. As soon as the animal entered the laboratory, a typical seizure occurred, similar in character to those noted previously. While this dog had been kept in the dog house, no convulsive reactions had ever been noted. Inasmuch as gliadin was being prepared in the adjoining laboratory, the seizure was suspected of being allergic in character. In this connection it is pertinent to point out that the initial toxic reactions, observed with the dogs subsisting on high-gliadin diets, occurred at the time when the animals were being fed.

Evidence that idiopathic epilepsy may be a sensitization disease has been advanced by Howell ('23) and Miller ('24). Since the symptoms observed in our animals were not typical of anaphylactic shock in the dog (Melnick, Burack and Cowgill, '36), we had neglected previously to investigate the possibility that the epileptiform reactions may be due to the dogs having been sensitized to gliadin. Subsequent investigations were conducted in order to test the validity of such a hypothesis.

[13] We are indebted to Dr. Harry M. Zimmerman of the Department of Pathology, Yale University School of Medicine, for interpretations of the kidney sections.

[14] We acknowledge our indebtedness to Dr. Louis Weinstein of the Department of Bacteriology, Yale University School of Medicine, for his generous assistance during this phase of the investigation.

Preliminary tests, passive transfer skin tests on a non-sensitized dog and the intravenous injections of a gliadin solution into guinea pigs which had been passively sensitized with the serum from dog no. 10, suggested that the toxicity of the high-gliadin diets may be due to a protein sensitization. The protein was employed as a 4.4% solution in 75% alcohol.[15] It is not considered advisable to inject intravenously such a solution since, in addition to the toxicity of the strong alcoholic solution, the gliadin is immediately precipitated on coming into contact with the blood. However, if an equal volume of a $\frac{N}{10}$ sodium hydroxide solution is first added to the gliadin solution, the further addition of 8 parts of a 0.5% sodium chloride solution does not precipitate the protein. In the subsequent tests all the gliadin solutions used for intravenous injections were prepared according to this procedure. The apparatus and technique used for the injection of the solution were the same as that employed for the reinjection of the red blood cell suspensions into our dogs subjected to plasmapheresis (Melnick and Cowgill, '36). This point is mentioned since toxic reactions are frequently associated with intravenous injections due simply to faulty technic. We do not believe such an explanation can be offered for our observations.

A passive transfer test was conducted using a normal non-sensitized dog as the test animal. Dog no. 10 was bled 160 cc.; to this blood a 3% sodium citrate solution was added until the final concentration of the anticoagulant was 0.3%. Approximately 90 cc. of the citrated plasma (75 cc. of whole undiluted plasma) were injected intravenously into dog no. 53, a male terrier, weighing 7.0 kg. Twenty-four hours later the diluted gliadin solution, prepared according to the directions given above, was injected into the jugular vein at the rate of approximately 50 cc. per minute. Respiration and the heart rate were slightly stimulated. After 80 cc. of the solution were injected, the animal was given a rest period of 3 minutes; this was then followed by the injection of an additional 80 cc. During the second injection, there was a marked fall in the blood

[15] Analyses of the protein indicated a purity of 84.1%, so that the true concentration of gliadin in the alcoholic solution was actually 3.7%.

pressure, labored respiration and a pronounced cardiac arrhythmia. Almost simultaneously with the conclusion of the injection, respiration and the heart beat stopped. The injection of 0.25 cc. of an adrenalin solution [16] into the heart resulted within 1 to 2 minutes in the complete recovery of the animal.

The above experiment was repeated on another dog, but this time the animal, dog no. 54, a male terrier also weighing 7.0 kg., was not previously sensitized by plasma injections from dog no. 10. The response obtained with this animal resembled that recorded for dog no. 53. However, respiration and the heart beat did not cease; the animal recovered quickly without therapy of any kind.

It seemed to us that the differences in the degree of shock noted in the above experiments could be differentiated simply on the basis of dose. To test such a hypothesis, dogs nos. 10, 11 and 13, presumably actively sensitized to gliadin, were given an intravenous injection of the diluted protein solution. The doses, which were also given in two injections, were calculated on the basis of body weight. Thus, dog no. 10 received approximately 210 cc.; dog no. 11, 180 cc.; and dog no. 13, 210 cc. Precisely the same response, as that noted in the experiment conducted with dog no. 53, was obtained with dog no. 10. The intracardiac injection of adrenalin caused complete recovery from the shock reaction. In the case of dog no. 11, it was deemed advisable to allow more than 3 minutes between injections so as to compensate for any possible latency in the appearance of shock. Within 5 to 6 minutes after the first injection of 90 cc. of solution, the dog vomited and defecated; this was followed by complete prostration similar in character to that recorded with dog no. 54. The animal finally recovered without therapy and appeared quite normal 15 minutes after the injection. The injection of the remaining part of the dose resulted in no obvious harmful effects other than slight intoxication. Dog no. 13 failed to show any response to

[16] Adrenalin chloride (1: 1000), obtained from the Parke, Davis and Co., Detroit, Mich.

the injections other than symptoms of mild intoxication, such as a mild ataxia of about 10 minutes' duration.

If we accept the hypothesis that the toxicity of high-gliadin diets is due to a protein sensitization, the results obtained with dog no. 11 are easily explained. Apparently the initial injection of the protein solution more or less completely saturated the antibodies with the antigen. Thus the second injection was followed by no adverse effects, although the amount of foreign protein injected into the animal was doubled. As was pointed out in table 1, dog no. 13, in contrast to the other animals, exhibited only the mild, toxic reaction when fed the high-gliadin diet. Thus, the negative response to the intravenous injection of the gliadin solution in the case of this animal may not be inconsistent with the results recorded with dogs nos. 10 and 11. However, when the results obtained with this animal are compared with the observations recorded with dog no. 54, which had never subsisted on the high-gliadin diets, it appears that dosage may play some part in determining the character of the response to the intravenous injections of the protein solution here described. This conclusion is also supported by the fact that dog no. 10, presumably actively sensitized to gliadin, required a dose comparable to that injected into dog no. 53 before shock was manifest.

Although dog no. 54 showed symptoms of shock similar in character to those recorded with dogs nos. 53 and 10, the animal quickly recovered without the aid of adrenalin therapy. The possibility existed that, if a greater volume of the gliadin solution had been injected, the shock reaction would have been fatal. This was confirmed by the injection of a relatively large amount of the protein solution into another normal dog. This animal, dog no. 55, a female mongrel with an optimal weight of 12.6 kg., received 350 cc. of the gliadin solution intravenously. This dose in terms of body weight was approximately 20% greater than those administered to the other dogs. The first part of the injection was tolerated satisfactorily. After the usual 3-minute rest period the remainder of the dose was injected. The animal was thrown into profound shock.

In order to be sure that there would be no spontaneous recovery, we waited approximately 1 to 2 minutes before resorting to the use of adrenalin. Three 0.25 cc. injections failed to save the animal.

It would seem that the correlation of effect with dose should rule out anaphylaxis as the mechanism responsible for the shock reactions. However, it should be remembered that the solutions as injected contained only a small amount of protein, namely, 3.7 mg. per cubic centimeter. The possibility that some other factors might be responsible for the shock reactions was investigated. According to the studies conducted by Hyatt ('19), the alcohol could not be the cause of the appearance of shock. This was confirmed by injecting into each of the five dogs (nos. 53, 54, 10, 11 and 13) the alcoholic, alkaline, saline solution, prepared in the same manner as described but this time with no gliadin added. The volumes injected into each dog were actually 20% greater than those employed when gliadin was administered. These experiments were repeated a week later but this time casein [17] was injected instead of gliadin. It was concluded from these tests that the symptoms of profound shock noted in our dogs were due to the intravenous injection of the protein, gliadin, and not due to the character of the solvent or merely to the introduction of any foreign protein into the circulation.

In connection with these experiments, it is of interest to note that Seibert and Mendel ('23) found that most casein preparations, when injected intravenously into rabbits, cause a marked rise in body temperature, whereas gliadin is apparently without effect. However, the amounts of protein so administered were exceedingly small; for the most part only 5 mg. of protein were injected into each animal. Nevertheless, the effects obtained in the present study as a result of the gliadin injections cannot be explained on the basis of dose alone. It is true

[17] A 0.44% solution of a casein preparation obtained from the Arlington Chemical Co., Yonkers, N. Y., was used. This particular sample contained 13.72% nitrogen which is indicative of an 86.2% purity (Jones, '31). Thus the true concentration of the protein in the alcoholic, alkaline, saline solution was actually 3.8 mg. per cubic centimeter.

that the adjustment of the pH of the gliadin solution to that of the blood results in a precipitation of the gliadin in the form of extremely fine particles which settle out very slowly. Thus, it seems possible that such occurs when the protein solution comes in contact with the blood. It also appears likely that under such conditions the formation of multiple emboli may occur. However, it is impossible to attribute the observed toxic reactions to such a cause. Dog no. 11 showed a typical shock reaction after the injection of only one-half of the calculated dose of the gliadin solution. If this effect is to be explained on the basis of the formation of multiple emboli, one should expect a second reaction when the remainder of the dose was injected. However, the administration of the second half of the protein solution, after the animal had recovered from the initial shock reaction, resulted in no obvious untoward effects other than a mild intoxication. Furthermore, the rapid and complete recoveries of dogs nos. 53 and 10 from shock, following intracardiac administration of adrenalin, also preclude the possibility that the toxicity of the protein solutions is due to the tendency for the formation of multiple emboli.

It seems to us that the evidence presented suggests that the toxicity of high-gliadin diets may be due to a sensitization of the animal to the protein. It is not unlikely that all dogs are more or less sensitive to gliadin. It may be that the feeding of rations containing excessive amounts of this protein to the dogs finally results in the absorption of certain fragments of the gliadin molecule, which in sufficient concentrations are capable of precipitating the epileptiform reactions described in the early part of this paper.

In the case of the rat, the feeding of the high-gliadin diet produced no 'toxic' effects other than an impairment of growth and this was corrected by adding lysine to the ration. If the seizures observed with the dogs can be attributed to a protein sensitization, then the negative effects obtained with the rat are consistent with the fact that this species is particularly non-sensitive to the injection of foreign protein. This belief

was confirmed by tests conducted on nine mature rats. These animals were injected by way of the femoral vein with varying doses of the gliadin solution, calculated on the weight basis to be in some cases actually twice as large as that used in the study with the dog. On the basis of these tests, it was concluded that the rat is undoubtedly more resistant than the dog to the intravenous administration of gliadin, and that any toxicity evidenced by the rat is probably due simply to the character of the solvent used.

Many investigations, in which gliadin was used as the sole source of protein in the ration, have been reported in the literature without any mention of a toxicity having been observed. This may be due to the fact that in all such experiments, with which we are familiar, the rat was used as the experimental animal. This species has been shown in the present study to be particularly resistant to the toxicity of high-gliadin diets. In the case of the dog both the cost of feeding the expensive gliadin rations to such a large animal and the fact that most dogs exhibit difficulty in consuming such diets have led investigators to refrain from using this species in feeding experiments involving the use of gliadin. During the ingestion of such rations this protein tends to 'gum up' and stick to the teeth. However, as we discovered (Melnick and Cowgill, '37), if the diet is first mixed with an equal part of water to form a loose pasty mass, no difficulties will be encountered in getting the dog to ingest its daily aliquot of the ration.

SUMMARY

In the present study it was possible to produce convulsive reactions in all of six dogs fed high-gliadin diets in which gliadin was the sole protein and furnished 16% or more of the caloric intake. Intestinal parasites were ruled out as possible cause of the toxic reactions. The fact that there was an initial lag in the appearance of gliadin toxicity and a delay in the disappearance of the seizures suggests the accumulation of some toxic substance during the gliadin feedings and its subsequent elimination. The increases in blood non-protein nitrogen were not sufficiently large to indicate that the con-

vulsions were uremic in nature. Various experiments tentatively suggest that this toxicity of the high-gliadin diets may be due to a protein sensitization.

The gliadin, isolated from a 75% alcoholic extract of gluten flour, contained only 0.15% ash. Selenium was not present as a contaminant of the protein.

Insofar as growth data are concerned, feeding experiments showed that the gliadin was free of any substance toxic for the growing rat. No convulsive reactions were observed in the rats subsisting on a gliadin diet in which the protein furnished as much as 25.6% of the caloric intake. Apparently susceptibility to gliadin toxicity is a species characteristic.

The rats, fed the 36% (by weight) gliadin diet, subsisted on a ration containing actually 13.2% glutamic acid. The ingestion of a dietary mixture, containing such a high concentration of this amino acid, appeared to be non-toxic for this species.

The nutritional significance of the lysine present in gliadin was emphasized. Rats fed an 18% or a 36% gliadin diet were stunted practically to the same extent. However, when both rations were supplemented with the same amount of lysine, growth was twice as rapid in those animals fed the 36% diet.

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THE EFFECTS OF DEFICIENCY OF PHOSPHORUS ON THE UTILIZATION OF FOOD ENERGY AND PROTEIN [1]

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INTRODUCTORY

A continuing program of studies is in progress, at the Institute of Animal Nutrition of Pennsylvania State College, in which is sought the reaction of growing albino rats to deficiencies of single nutritive principles; the experimental procedure, which is original to this series, being the same in each unit.

The characteristics of this procedure, which is called 'the body balance method,' are that the complete accounting for the disposal of the food energy is accomplished as follows: 1) The energy of the body gain is measured as the difference between the energy of the bodies of two groups of animals, one of which has received an experimental diet, and the other has been selected to represent the experimental group in its initial status; 2) the energy of the excreta of the experimental group is directly determined; and 3) the heat production is measured as the gross energy of the food minus the energy of the excreta and of the body gain.

The mathematical validity of this procedure is safeguarded especially by the employment of large numbers of

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[2] This paper is based on experiments, conducted under the author's direction, by Orme J. Kahlenberg, R. W. Swift, Alex Black, Winfred W. Braman (deceased) and Max Kriss.

experimental subjects, fed for an extended period of observation; and when it is used as the basis of comparison of the energy values of diets, these are fed with the paired method of control.

Consideration of the nature and of the constancy of the data on which this procedure is based show that, as a method of measurement of the total, unfactored heat production, it is characterized by a high degree of validity.

An incident in the current trend of thought relating to the broader economic problem of nutrition, as resulting from new trends of the times, is that minimum nutritive requirements of man, and of domestic animals, have lost much of their former apparent importance; while optimum nutritive requirements have attained new practical significance.

In other words, a new economy of superabundance is superseding an age-old 'economy of minimum;' and in this relation it is pertinent to investigate the questions as to what are the limits of useful phosphorus intake, for the growing albino rat, and what are the evidences of phosphorus deficiency.

To specify the functions of phosphorus in animal metabolism is to enumerate the vital processes of the body; the integrity of these processes being maintained by safety provisions of various kinds, including stores of phosphorus, mainly in the skeleton, of extents commensurate with the demands likely to be made upon them, and also with the supreme importance of the animal economy as a whole.

By virtue of these safeguards, the vital processes of the organism are insured against disturbance from variations likely to occur in the phophorus intake; and, within limits, such variations are presumably of consequence to the animal only as causing fluctuations in the body reserves of phosphorus—without effects on the vital processes. These safeguards, however, may be overtaxed—with the result that metabolism is affected.

The effects of phosphorus deficiency, therefore, may be any such disturbance of metabolism as is precipitated—immediately or later—by insufficient intake of this element.

In view of the conception of complete nutrition, and the obvious facts that no essential nutrient can be more essential than another; but that there are inevitable differences in the relative extent of the body reserves of essential nutrients, and in the rapidity with which they may be physiologically mobilized; the noticeable effects of differences in the level of phosphorus supply may not be definitely specific, in the sense of being obviously related either to its chemical nature or to the more conspicuous of its functions, but may be general in character. In fact, in a critical sense, there is no such thing as phosphorus metabolism; there is only metabolism in general, in which phosphorus is involved—in a vast diversity and complication of ways.

In the conduct of this series of experiments, therefore, the observations made are related to each other only as results of studies of single nutritive deficiencies, all having been obtained by the same method.

The background of the present study may be considered to be the vast existing knowledge of the fundamental relations of phosphorus in the metabolism of the tissues and organs of the body; but this investigation itself deals only with considerations of phosphorus deficiency in the albino rat, as affecting the economy of utilization of food energy and protein for maintenance and body increase.

Numerous students of animal feeding have drawn conclusions of fundamental implication, as to the effects of phosphorus deficiency, from simple, incompletely controlled feeding experiments. In many cases the results seem so obvious as to render superfluous the more critical investigation of the subject; but attempts to determine the facts more certainly have shown that very few experiments, in the field specified, have been conducted with sufficiently critical control to demonstrate fundamental truths; and that there is need for further investigation by intensive methods—especially to answer questions as to the degree to which the phosphorus intake may be varied without disadvantage, and as to the ways in which, and the extents to which, phosphorus

deficiency may affect the utilization of food energy and protein. With these considerations the present study is concerned.

The scope of this investigation and the character of the findings, seem not to justify a general review of the literature of phosphorus metabolism, but a few of the later studies, which throw light on the subject as considered, will be mentioned.

Phosphorus deficiency occurs much more commonly with cattle than with other species; and, in this relation, is of economic importance, in the United States, in certain more or less definitely delimited regions characterized by low-phosphorus soils, in California, Florida, Kansas, New Mexico, Michigan, Minnesota, Wisconsin and Texas.

Theiler, Green and Du Toit ('24) concluded, from feeding experiments, that there was a lowered economy of feed utilization in aphosphorosis.

Eckles and Gullickson ('27) found that a plane of nutrition 1.2 times normal was required to maintain live weight in certain phosphorus deficient cows.

Woodman and Evans (30) observed that a deficiency of calcium and phosphorus in the diet of sheep did not depress the digestibility of the ration.

Eckles, Becker and Palmer ('26), and Eckles, Gullickson and Palmer ('32) concluded, from studies of phosphorus deficiency in cattle, by means of feeding experiments, that

> under extreme conditions, as in the area studied, a shortage of phosphorus becomes a limiting factor in the economical utilization of feeds, and in the growth of cattle. The data reported bear out the generally accepted fact that phosphorus is involved in the intermediary metabolism of carbohydrates.

The most inclusive studies that have been made of the effects of phosphorus deficiency on the performance of cattle are those of Riddell, Hughes and Fitch ('33, '34 a, b). Their observations, confirming much that had been learned by others, but also covering extensive new findings, may be summarized as follows: Phosphorus deficiency in dairy cows

causes failure and depravement of appetite, stiffness and muscular weakness, failure of nutrition as evidenced by loss in weight and excessive maintenance requirement, low inorganic phosphorus in the blood, failure of oestrum, low phosphorus but high calcium in the urine, negative phosphorus and calcium balances, rapid and shallow breathing, and increased pulse rate. There was no effect of phosphorus deficiency on body temperature, or on the digestibility or the metabolizability of the food; milk production was maintained at a relatively high level, in spite of phosphorus deficiency, and the animals on phosphorus deficient diet responded to dosage with monosodium phosphate by increase in appetite, in live weight, in lactation, and in improvement in the various pathological conditions resulting from phosphorus deficiency.

Riddell, Hughes and Fitch concluded, as had others previously, that, in consideration of the various conditions noted, phosphorus deficiency must cause increased heat production. They then measured the heat production of milking cows, as affected by phosphorus deficiency, by indirect calorimetry. The heat was computed from three 6-minute measurements of the oxygen consumption of the animal, 12 hours after feeding, by assuming a respiratory quotient of 0.82, the subject being in the lying position, which it had been taught to assume by 3 months' training.

The authors found a somewhat greater heat production with the phosphorus-deficient cows than with the controls. However, in consideration of the circumstances stated—especially that the total time of oxygen measurement was only one-eightieth of the day; and that these measurements were made 12 hours after feeding, and in only one position—which the animals took only as a result of long training—the competency of these oxygen measurements to indicate the effect of the phosphorus deficiency on the heat production seems questionable.

A recent paper by Robbins ('35) reports that with a 37% increase in the basal metabolic rate, under the influence of dinitrophenol, there was no significant change in the excretion of calcium, phosphorus and nitrogen. This was taken to

signify that increased metabolism of the body, as a whole, is not necessarily shared by the skeleton—at least in so far as changes in the intrinsic metabolism of the bones are measurable by alterations in the total exchange of the bone salts.

Aubel, Hughes and Lienhardt ('36 a, b) conducted experiments in a study of the effects of the plane of phosphorus intake on the gain in weight and body measurements of swine, the food consumption being maintained the same, in the high-phosphorus and the low-phosphorus groups, by permitting the pigs to have water only as it was consumed mixed with the feed.

From this study the authors concluded that the following abnormalities may result from the feeding of low-phosphorus rations: 1) a lowering of the inorganic phosphorus in the blood, 2) a failure of normal growth and development of bone and muscle, 3) a poor utilization of feed and storage of energy, 4) a loss of appetite, and 5) a marked increase in thirst, and a corresponding excretion of urine.

In speaking of the effects of phosphorus deficiency on the kidneys the authors said,

> Microscopically this organ showed evidence of a chronic diffuse nephritis of the parenchymatous type, and presented widened glomerular spaces around the glomerular tufts, and also widely distended uriniferous tubules with flattened epithelial cells.

The conclusion as to the utilization of food and storage of energy rested upon live weight data, and other observations, without body analyses or chemical study of metabolism.

Aubel and associates ('36 a) call attention to the fact that in the experiments of Riddell, Hughes and Fitch phosphorus deficiency in cows led to increased water intake, and increased outgo of urine.

Kleiber, Goss and Guilbert ('36) conducted a feeding and energy metabolism study of phosphorus deficiency as affecting beef heifers. There was an unfavorable effect of phosphorus deficiency on the appetite; a decrease in the inorganic phosphorus of the blood; but no effects on the body

temperature, the digestibility and the metabolizability of the food energy, and the fasting katabolism.

These authors concluded that phosphorus deficiency decreased the efficiency of food protein to spare the katabolism of body protein; and decreased the efficiency of utilization of food energy consumed in excess of the maintenance requirement.

From the data presented it is not possible to judge as to the validity of the important conclusion last stated; in fact, though food phosphorus must be necessary to the normal utilization of food energy and protein, in accord with the prevailing understanding of the fundamental physiology of nutrition, there remains a decided lack of clear-cut evidence that variations in the phosphorus content of the diet, under conditions of practical nutrition, affect the efficiency with which the food is utilized.

The investigation to be discussed comprised two experiments, which will be designated experiment 1, and experiment 2, the details of technic in both being as outlined by Swift, Kahlenberg, Voris and Forbes ('34). In both experiments comparisons were made of two rations differing only in phosphorus content, by means of 10 weeks' growth, metabolism and body analysis studies, in which the feed was apportioned by the paired litter-mate feeding method.

EXPERIMENT 1

The subjects were twelve pairs of weanling albino rats; six pairs being males, and six pairs females.

The basal ration consisted of wheat gluten 14%, casein (vitamin-free) 8%, Crisco 10%, dextrin 59.16%, salt mixture xxx 2.60% and cod liver oil 5%. The salt mixture was a modification of Osborne and Mendel's formula, free from calcium and phosphorus (Sherman and Smith, '31). In order to have a proportion of calcium equivalent to that found in synthetic rations including the usual Osborne and Mendel complete salt mixture, 1.24% of calcium carbonate, c.p., was added to one ration, and 1.28% tricalcium phosphate, c.p.,

to the other ration. In the phosphorus-supplemented ration the dextrin was reduced to 59.12%. Vitamins B and G, and additional cystine, were furnished to all rats by feeding 0.20 gm. of dried brewer's yeast daily.

The supplemented ration contained 0.366%, and the phosphorus deficient ration of 0.137%, of phosphorus.

Thus an average weekly food consumption of 40 gm. of the basal part of the low-phosphorus ration furnished 54.8 mg. of phosphorus; and, with 1.4 gm. of yeast (2.49% phosphorus), the deficient rats obtained a total of 89.7 mg. of phosphorus per week.

A diet fed for 8 or 9 days at the beginning of the experiment, but which proved unsatisfactory, and was therefore changed, differed from that above specified in that blood albumin was used in the place of the casein. The ration was analyzed, and was accounted for in the computation of the intake of phosphorus, protein and energy.

At the end of the experiment, the rats were killed, and x-ray photographs were taken of one hind leg of each individual. The bodies were then analyzed for moisture, ether extract, nitrogen, energy and phosphorus.

In view of the fact that the results corresponding to the two diets were essentially the same in regard to so large a proportion of the observations made, the numerical findings are presented only in summary form, as in tables 1 and 2.

After the termination of this experiment a special test of the palatability of the diet used was made by means of five pairs of rats, fed ad libitum, with measured feed intake and live weight, during 5 weeks. During 4 of the 5 weeks the rats ate more of the deficient than of the supplemented ration, and the total amount of the deficient ration eaten was 6.8% more than that of the supplemented diet, the natural inference being that the calcium phosphate served to diminish the palatability of the diet in which it was fed.

DISCUSSION OF RESULTS

In this experiment there were 358 refusals of feed, 206 being by the animals on the phosphorus supplemented diet. This deviation of 27.0 from the mean, if chance only determined the result, is 2.85 times the standard deviation, 9.46, and would occur by chance once only in 230 trials. The phosphorus-supplemented ration, therefore, limited the food consumption of the rats on the phosphorus deficient ration.

The average gains in body weight (contents of alimentary tract removed) of the rats on the phosphorus deficient and phosphorus supplemented rations were 90.15 gm. and 89.87 gm., respectively, this difference not being statistically significant. The growth for the 10 weeks was fairly satisfactory.

The fact that the supplemented diet was the limiting factor in relation to food consumption, and that there was practically no difference in the live weights of the rats fed the two rations, suggests that the phosphorus content of the basal diet, low though it was, was sufficient to satisfy the requirements of the fundamental physiological functions.

The data presented in table 1 show that the difference between the phosphorus contents of the diets (0.137 and 0.366%) produced a highly significant difference between the phosphorus contents of the bodies of the two groups (0.94 and 1.08%); but that this difference in food phosphorus produced no statistically significant difference between the phosphorus deficient and the phosphorus supplemented groups with reference to any other of the important observations indicating the relative economy of utilization of food energy and protein.

The x-ray photographs revealed no discernible difference in the bones of the two groups, though the skeleton was doubtless the seat of the greater part of the difference in the phosphorus contents of the bodies; and these photographs also revealed the fact that the phosphorus deficient rats were not rachitic.

EXPERIMENT 2

In this experiment efforts were made to obtain better food consumption and growth by changes relating to both rats and diets; thus ten pairs of male, pied rats were used instead of albinos; the rats were allowed to attain a weight of about

TABLE 1

Comparative utilization of food by rats on phosphorus deficient and phosphorus supplemented diets

	PHOSPHORUS DEFICIENT DIET	PHOSPHORUS SUPPLEMENTED DIET	ODDS THAT DIFFERENCE IS SIGNIFICANT
1. Ratio of gain in weight to dry matter of food	25.4	25.4	..
2. Energy of body gain as percentage of food energy	11.6	11.0	4 to 1
3. Food nitrogen retained, per cent	26.7	27.2	2 to 1
4. Energy gained as fat, per cent (of gain)	43.7	40.5	9 to 1
5. Energy gained as protein, per cent (of gain)	56.3	59.5	9 to 1
6. Digestibility of food protein, per cent	95.3	95.1	12 to 1
7. Digestibility of food energy, per cent	96.3	96.3	..
8. Heat production as per cent of food energy	81.1	81.9	15 to 1
9. Phosphorus content of rat bodies, grams	0.94	1.08	10,000 to 1

NOTE: All data in the above table are averages of individual values representing twelve rats.

120 gm. before the experiment was started, whereas in experiment 1 the albino rats were used as at weaning; and, in relation to the diets, the wheat gluten component was diminished from 14% to 6%; 8% of casein was replaced by 12% of lactalbumen; 10% of Crisco was replaced by 15% of butterfat; and a vitamin B concentrate was added to the diet.

The components of the diets were as follows:

	Phosphorus deficient	*Phosphorus supplemented*
Wheat gluten	6.0	6.0
Lactalbumen	12.0	12.0
Dextrin	60.7	60.7
Butterfat	15.0	15.0
Salt mixture	2.6	2.6
$CaCO_3$	1.4	1.4
$NaHCO_3$	2.7	..
Na_2HPO_4	..	2.3
	100.4	100.0

In addition, vitamin-containing amendments, as follows, were fed apart from the remainder of the diet, each day, to the rats of both groups: cod liver oil 12 drops (0.35 gm.); Harris yeast concentrate 1 tablet (0.1107 gm.), and diluted vitamin B concentrate[3] 12 drops (0.53 gm.; equal to 4 drops vitamin B complex liquid, type 1; equal to 0.1767 gm. dry matter).

The potency of this vitamin B complex was given by the manufacturer as B_1, 40 Chase and Sherman units per cubic centimeter, and B_2, 6 Sherman and Borquin units per cubic centimeter. This was fed in a 1 to 2 (H_2O) dilution.

The phosphorus deficient diet contained 0.133%, and the phosphorus supplemented diet 0.653% phosphorus—on an average—the percentages differing slightly for the different individuals, since the vitamin supplements were fed in constant quantities separate from the remainder of the diet.

The effective palatability of the diets, as indicated by the refusal of feed, as the feed for each pair was increased until one individual refused, was, in this experiment, in favor of the supplemented diet. In eight among the ten pairs of rats the individual which received the supplemented diet refused feed a smaller number of times than did its pair mate; and among a total of 357 refusals of feed there were 124 with the supplemented diet, and 233 with the deficient diet. Whatever the influence of the calcium phosphate as an unpalatable component of the diet in the first experiment, therefore, this was

[3] Vitab Products, Inc., San Francisco.

counterbalanced by the effects of the several changes that were made in the diet between the first and the second experiments.

During the course of the experiment it was observed that the feces from the deficient diet were much darker in color than those from the supplemented diet, and rat no. 5 passed bloody urine, after having been on the deficient diet 19 and 20 days. As the animals were killed, at the end of the experiment, the contents of the intestines of the deficient rats were, as a rule, much darker than were those of the phosphorus-supplemented rats. The small intestines of the deficient rats were black, as compared with the dark, yellowish gray intestines of the supplemented rats; and the large intestines of the former were also nearly black, as compared with the normal, dark greenish color of the intestines of the supplemented rats.

Also, with rats nos. 1, 3, 5 and 9 one kidney was enlarged, and soft, and contained cheesy products of tissue destruction; while in rats nos. 1, 3 and 5 the liver was small, and dark in color. The one abnormal kidney in a supplemented rat was in no. 16. In this case the medulla had completely degenerated—only the cortex remaining. The significance of these observations was not determined.

During the first 4 weeks the rats were fed once a day; but, beginning with the fourth week, they were fed twice daily, with the hope that this would lead to increased food consumption.

The basal metabolism was determined by the Haldane open-circuit method, once, on each rat, during the quiet part of 5- or 6-hour intervals between 8.00 or 9.00 o'clock in the morning and 2.00 or 3.00 o'clock in the afternoon, between the sixth to the eighth weeks of the experiment.

The numerical data, as presented in table 2, reveal significant differences between the groups of rats on the two diets only with reference to the digestibility of the food protein, which was the higher with the rats on the deficient diet, and with reference to the phosphorus content of the bodies of the rats.

While the effect of the phosphorus supplement to depress the digestibility of the protein of the diet was slight, this tendency was manifest in nine among ten pairs of rats, and the odds were 216 to 1 that the digestibility of the protein of the supplemented diet was less than that of the deficient diet. However, since the food consumption of each pair of rats

TABLE 2

Comparative utilization of food by rats on phosphorus deficient and phosphorus supplemented diets

	PHOSPHORUS DEFICIENT DIET	PHOSPHORUS SUPPLEMENTED DIET	ODDS THAT DIFFERENCE IS SIGNIFICANT
1. Ratio of gain in weight to dry matter of food	9.6	10.1	3:1
2. Energy of body gain as percentage of food energy	5.4	6.2	4:1
3. Food nitrogen retained, per cent	17.9	18.8	2:1
4. Energy gained as fat, per cent (of gain)	35.7	40.8	4:1
5. Energy gained as protein, per cent (of gain)	64.3	59.2	4:1
6. Digestibility of food protein, per cent	93.1	92.3	216:1
7. Digestibility of food energy, per cent	95.5	95.2	18:1
8. Heat production as per cent of food energy	85.6	86.2	4:1
9. Basal metabolism, calories per 100 gm. body weight	575.5	553.7	18:1
10. Phosphorus content of rat bodies, grams	0.98	1.16	2499:1

NOTE: All data in the above table are averages of individual values representing ten rats.

was determined by the individual that desired the smaller quantity, the food intake of the ten pairs was not exactly the same, and the two groups of digestion coefficients compared were not exactly homogeneous as to significance.

The range of variation in the digestibility of the protein of the two diets was as follows: for the deficient diet, 91.8 to 94.2%; and for the supplemented diet 90.6 to 93.2%.

The phosphorus content of the bodies of the rats differed consistently in accord with the phosphorus contents of the diets, the average difference being 18.37% of the quantity found in the bodies of the rats on the phosphorus deficient diet.

SUMMARY

The effects of phosphorus deficiency on the growing rat were studied in two 70-day body balance and metabolism experiments conducted with paired feed control.

In the first experiment the phosphorus deficient and the phosphorus supplemented diets contained 0.137% and 0.366% of phosphorus, respectively, on the dry matter basis; and after two groups of rats had consumed these diets for 70 days the corresponding average phosphorus contents of their bodies were 0.94% and 1.08%, respectively, the difference between these values being 15% of the lower one.

In the second experiment the low and the high phosphorus diets contained 0.133% and 0.653% of phosphorus, respectively; and after 70 days' feeding on these diets the corresponding average phosphorus contents of the rats' bodies were 0.98% and 1.16%, respectively, the difference between these values being 18% of the lower one.

In the first experiment, the difference in dietary phosphorus intake which produced a 15% difference in body phosphorus produced no observed difference in growth or in the utilization of food energy or protein.

In the second experiment, the difference in phosphorus intake which produced an 18% difference in body phosphorus produced a slight but statistically significant depression in the digestibility of food protein. This was the effect of the disodium phosphate of the high-phosphorus diet.

In this second experiment there were no other observed effects of the difference in phosphorus intake on the utilization of food energy or protein, or on growth.

The phosphorus content of the low phosphorus diet was as low as the experimenters could make it and at the same time have sufficient feed consumption and growth to serve the purposes of the investigation. Normal growth could not have been obtained on a diet still lower in phosphorus.

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THE COMPARATIVE NUTRITIVE VALUES OF GLUCOSE, FRUCTOSE, SUCROSE AND LACTOSE WHEN INCORPORATED IN A COMPLETE DIET

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There are many indications in the literature that the various sugars, ingested separately and without admixture with other nutrients, are absorbed at different rates, are utilized in different ways quantitatively, and exert different physiological effects. They differ in the rate with which they are oxidized or are converted into glycogen or into fat, or in the extent to which they are utilized in various ways. Their specific dynamic effects, either the total effect, the peak effect, or the time curve, have been observed to be somewhat different, and the sugars seem to vary also in ketolytic and protein-sparing capacity. While the experimental evidence on these various points is not always concordant, the fact that significant differences exist is clear.

In its favorable effect upon the absorption and utilization of calcium and phosphorus, lactose stands apart from all other sugars, as the work of Bergeim ('26), Kline, Keenan, Elvehjem and Hart ('32), and of Robinson, Huffman and Mason ('29) in particular prove. This favorable effect seems to be a result of the tendency of lactose to maintain a higher hydrogen ion concentration throughout the intestinal tract by favoring the propagation of an aciduric flora, though other factors also seem to operate in this direction.

But the fact that sugars when fed alone exhibit individual effects does not imply that when fed as components of a well-balanced diet they will still react differently on the body or be utilized in different ways or to different extents within it, except as these individual effects relate to conditions obtaining in the intestinal tract. What the cells and tissues of the body do to a sudden influx of a particular sugar from the intestinal tract may not determine what they will do to the same sugar accompanied by all other nutrients needed for the maintenance of life, the promotion of growth and the performance of secretory functions. In the former case, in which sugars are fed alone, the disposal of the sugar may follow the path of least resistance, determined by the constitution of the particular sugar fed. In the latter case, with the possibility of manifold anabolic reactions, the same sugar may be used in a more purposeful manner, since its energy and its substance may now be utilized in the promotion of syntheses and interconversions of other nutrients. It is not inconceivable that the various sugars, within certain ranges of dietary concentration at least, are of identical value when incorporated in a diet thus providing all of the nutrients essential for all prevailing body functions. The expectation that the effects of nutrients determined by the feeding of each separately, are necessarily summated when they are combined in a balanced diet, is fundamentally unsound and demonstrably erroneous in many instances, for example, their specific dynamic effects and net energy values.

A number of investigations have been concerned with the nutritive evaluation of balanced diets differing only in the kind of carbohydrate contained in them. Unfortunately for the purposes of this discussion, most of these experiments involved no control of the amounts of food consumed by comparative animals and, for unascertained reasons, the rate of food consumption has quite often differed markedly among different experimental groups. The interpretation of the differences in the rates of gain (or in the composition of the gain) observed, in terms of differences in the inherent nutritive value of the sugars, is difficult if not impossible. Even

when the differences in food intake among comparative animals with unrestricted access to food happen to be apparently slight, the observed differences in experimental measurements may, partly as a result of the failure to control food intake, be so variable as to obscure possible small differences in the nutritive value of the sugars tested. Thus, in the experiments of Speirs and Sherman ('36), it was found that the amounts of calcium and phosphorus retained in the bodies of rats were not statistically different whether they were grown upon diets containing one-third glucose, or dextrin, or cornstarch, or sucrose, or a spray-dried corn sirup containing a mixture of glucose, maltose and dextrin. However, the variations observed in the mineral retentions of the various groups of experimental rats were such that it is impossible to distinguish statistically between the calcium or phosphorus balances of the male rats on the basal diet alone, and those of the male rats on the basal diet diluted with one-half its weight of the various carbohydrates tested. The latter diets contained one-third less calcium and phosphorus than the undiluted basal diet. With the female rats significant differences were observed in only seven of the ten comparisons between the basal diet and the sugar diets.

The experiment of Feyder ('35) comparing the nutritive values of sucrose and glucose was planned according to the paired-feeding method. The sugars constituted 68% of the experimental diets, which were adequately provided with protein, minerals and the vitamins contained in cod liver oil and yeast. The rations were carefully equalized with respect to energy, and at the termination of the experiment, after 16 to 29 weeks of feeding, seven pairs of the surviving rats were killed and their composition compared with that of control rats sacrificed at the beginning of the experiment. Under the conditions of this well-controlled experiment, the sucrose diet promoted more rapid gains in weight than the glucose diet, principally because of a more rapid deposition of fat, but also because of a significantly greater deposition of protein. The formation of glycogen was not significantly different between

the two groups of rats. Unfortunately the experimental rations were not completely balanced, apparently, according to the author, because of a deficiency in unsaturated fatty acids. Most of the rats, sometime during the feeding period, showed tail and skin symptoms similar to those described by Burr and Burr ('29) as evidence of a fat-deficiency disease.

In controlled feeding tests with rats and pigs, Whittier, Cary and Ellis ('35) compared the nutritive values of lactose and sucrose, using rations containing 30% (rats) or 40% (pigs) of sugar. In the rat experiments, in which ten pairs of animals were used, the rate of growth on the two experimental rations was practically identical, as was also the chemical composition of the carcasses as far as may be judged from the analyses of only two pairs. In the pig experiments, twelve animals were fed in four groups of three each; one pig in each group received the lactose ration, one the sucrose ration and one a 'control' ration the carbohydrate of which was derived entirely from brewer's rice. The pigs were fed individually and those in each triplet were fed the same amount of ration, determined by the pig consuming the least. During a feeding period of 25 weeks, the average daily gains in body weight averaged 0.68 pounds for the control pigs, 0.54 pounds for the sucrose pigs and 0.52 pounds for the lactose pigs, but the differences in rate of gain among the different groups are not statistically significant. Also the chemical analyses of carcasses revealed a higher fat content and a lower protein content of the pigs on the sucrose ration as compared with those on the lactose ration, though only three of the sucrose pigs were analyzed. The slow gains of the pigs suggest that the rations may not have been properly balanced.

Neither Feyder nor Whittier, Cary and Ellis consider the possibility of a difference in the digestibility of rations containing different carbohydrate components.

The experiments reported in this paper are concerned with a comparison of the nutritive value of glucose and sucrose, glucose and fructose, and glucose and lactose.

DESCRIPTION OF THE EXPERIMENTS

The experiments were performed upon growing rats, which were fed in accordance with the paired-feeding technic. During the feeding period, one or more determinations of the digestibility of the experimental rations were made, and in one comparison (glucose versus sucrose) qualitative tests were made for the presence of sugar in the urine. In the comparison of glucose and fructose, the paired rats were put in revolving cages, equipped with revolution counters, for periods of 6 to 8 weeks for the purpose of detecting any differential effect of the experimental rations on the voluntary activity of the animals. At the termination of the feeding periods all surviving rats were sacrificed, the contents of the gastro-intestinal tract removed, the empty carcasses weighed, frozen solid and ground finely while still frozen. The samples thus prepared were analyzed for moisture, fat (ether extract), protein ($N \times 6.25$), ash, calcium and phosphorus (except for the rats in the comparison of glucose and sucrose). Also, the heat of combustion (gross energy) of all samples was determined in a Parr oxygen-bomb calorimeter. All gains in body weight were computed from the initial live weight, representing the average of weights taken on three consecutive days, and the final empty weight.

The experimental rations were planned to contain from 60 to 70% of the various sugars, to be adequately balanced, and to be equal in protein and energy. Their composition is given in table 1.

The sugars used in these rations were analytical reagent or C.P. grade, except that the fructose was of only 90% purity. They possessed the following heats of combustion expressed in calories per gram: glucose, 3.699, sucrose, 3.955, fructose, 3.704, and lactose, 3.702. At the time the experiment was undertaken, it was not suspected that the sugars would be likely to contain appreciable amounts of calcium or phosphorus, or that the experimental diets would be likely to exert a differential effect on the retention of these elements in the body. Since such differential effects did in fact appear, the

question arose as to whether they could have been the result of the presence of these minerals in the sugars in variable concentrations. The same or similar supplies of the sugars were therefore analyzed for calcium and phosphorus. No detectable amounts were present in the sucrose, and extremely minute amounts in the glucose (0.000008% phosphorus and 0.00042% calcium). The lactose contained 0.0010% phosphorus and 0.019% calcium, while the fructose contained 0.00027% phosphorus and 0.056% calcium. The latter contamination is equivalent to an intake of 3.4 mg. of calcium per 10 gm. of ration.

TABLE 1

Composition of rations

CONSTITUENTS	RATION 1 (SUCROSE)	RATION 2 (GLUCOSE)	RATION 3 (FRUCTOSE)	RATION 4 (GLUCOSE)	RATION 5 (LACTOSE)
Casein	18	18	..	..	..
Dried whole egg	..	..	25	25	25
Salt mixture	4	4	4.5	4.5	4.5
Cod liver oil	2	2	2	2	2
Yeast	6	6	6	6	6
Glucose	..	70	..	60	..
Sucrose	66.4	..	..	..	..
Fructose	..	..	60	..	..
Lactose	..	..	..	..	60
Agar	..	..	2.5	2.5	2.5
Water	3.6	..	..	..	..
Average calories per gram	3.956	3.969	3.990	3.978	4.018

The experimental rations contained about 0.68% calcium and 0.71% phosphorus.

THE RESULTS OF THE EXPERIMENTS

Glucose versus sucrose. The nutritive values of glucose and sucrose were compared with nine pairs of rats. The essential results of the feeding experiments and the carcass analyses are collected in table 2. In this comparison, ration 1 was compared with ration 2.

TABLE 2

The results of the comparison of glucose and sucrose

RAT NO. AND SEX	SUGAR TESTED	DAYS ON TEST	INITIAL BODY WEIGHT	FINAL EMPTY WEIGHT	GAIN IN WEIGHT	TOTAL FOOD CONSUMED	COMPOSITION OF CARCASS					
							Moisture	Protein	Fat	Energy content	Ash	Calcium
			gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*calories*	*gm.*	*gm.*
85 ♀	Sucrose	76	74	192	118	740	112.7	35.3	37.2	535	7.10	2.03
86 ♀	Glucose	76	70	202	132	740	122.5	39.5	34.5	520	7.35	2.06
87 ♂	Sucrose	83	73	271	198	1035	156.8	56.7	50.5	753	9.34	2.52
88 ♂	Glucose	83	68	277	209	1035	167.7	56.0	44.6	699	8.80	2.36
89 ♀	Sucrose	92	60	169	109	788	105.9	35.6	20.8	380	7.54	2.20
90 ♀	Glucose	92	55	174	119	788	101.5	33.2	33.8	483	6.33	1.72
91 ♂	Sucrose	69	69	263	194	835	156.8	51.7	47.5	706	8.77	2.41
92 ♂	Glucose	69	70	265	195	835	156.2	50.1	50.9	739	8.45	2.28
93 ♀	Sucrose	75	55	192	137	785	113.2	36.9	35.1	511	7.32	2.09
94 ♀	Glucose	75	58	191	133	785	119.7	38.6	26.4	442	7.06	2.04
95 ♀	Sucrose	69	64	193	129	718	116.8	37.6	32.2	492	7.47	2.18
96 ♀	Glucose	69	63	195	132	718	121.0	37.7	29.3	475	7.11	1.96
97 ♂	Sucrose	69	50	222	172	722	143.6	46.0	25.9	479	7.39	2.11
98 ♂	Glucose	69	52	214	162	722	124.7	40.3	45.3	614	6.52	1.75
99 ♀	Sucrose	69	48	170	122	669	102.5	30.8	29.4	448	6.24	1.90
100 ♀	Glucose	69	53	175	122	669	101.2	31.8	37.1	507	5.61	1.58
101 ♀	Sucrose	69	40	178	138	684	106.5	33.9	29.1	457	6.98	2.05
102 ♀	Glucose	69	41	167	126	684	100.9	32.4	26.9	436	6.13	1.70
Averages	Sucrose		59.2	205.6	146.3	775.1	123.87	40.50	34.18	529.0	7.572	2.166
	Glucose		58.9	206.7	147.8	775.1	123.93	39.94	36.53	546.1	7.040	1.939

On equal amounts of food, the gains in body weight were practically identical on the two rations. The contents of the carcasses of pair mates in moisture, protein and fat are not distinguishable statistically, and averaged very nearly the same for the rats on glucose and on sucrose rations. The gross energy contents of the carcasses of pair mates evidently were not affected by the difference in the carbohydrate components of the rations compared. However, with respect to ash and calcium deposited in the bodies of the experimental rats, the storage was greater in eight of the nine pairs for the rat on the sucrose diet. A statistical analysis of the paired differences according to the method of Student ('08) shows clearly that the differences must have been the result of the

TABLE 3

Average percentage composition of the glucose and sucrose rats

RATION	MOISTURE	PROTEIN	FAT	ASH	CALCIUM	ENERGY IN CALORIES PER GRAM
Sucrose	60.30	19.61	16.51	3.72	1.071	2.561
Glucose	59.92	19.28	17.67	3.43	0.947	2.642

difference in experimental treatment imposed. For the ash content, the mean difference between pair mates was 0.532 gm. in favor of the sucrose rat, the standard deviation of differences was 0.401 gm., and the probability of a fortuitous outcome only 0.0029. For the calcium contents, the mean difference was 0.227 gm., the standard deviation 0.155 gm., and the probability 0.0016. Both probabilities are so small that they may be neglected. The differences not being fortuitous, must have been caused by the difference in the composition of the diets, since the amounts consumed by pair mates were identical. In these tests, therefore, sucrose induced a more complete retention of ash and of calcium from diets presumably containing generous amounts of minerals, than did glucose.

The percentage composition of the empty carcasses showed essentially the same differences as the absolute composition. The averages only are given in table 3. Again, only the ash

($m = 0.292$, $s = 0.216$, $P = 0.024$) and the calcium ($M = 0.124$, $s = 0.086$, $P = 0.0030$) differences are statistically significant.

The urines of the experimental rats were tested qualitatively for reducing sugars, using Benedict's solution. When the collections were taken before feeding, no reducing sugars were indicated, but when taken for a few hours after feeding, the urines from the sucrose rats invariably contained reducing sugars while those from the glucose rats were still sugar free.

Digestion trials clearly indicated that the fecal waste from the sucrose rats was significantly greater than that from the glucose rats, consuming the same amount of food, with respect to dry matter, nitrogen and energy. In a final test made upon all nine pairs of rats the fecal dry matter averaged 14.2% greater for the sucrose rats, the fecal nitrogen 25.5% greater, and the fecal energy 17.9% greater. The differences were in the same direction in all pairs. However, the digestibility of the food was evidently good in all rats, since the coefficients for energy averaged 96.20 for the sucrose ration and 96.80 for the glucose ration. The differences in the digestibility of the rations were slight, though highly significant statistically.

Glucose versus fructose. The results of the comparison of glucose and fructose (rations 4 and 3) are summarized in table 4. On the same amounts of food glucose rats gained in weight faster than the fructose rats in seven of the eight pairs. The mean difference in total gain between pair mates was 8.63 gm., the standard deviation of differences was 6.24 gm., and the probability of a fortuitous result was only 0.0041. Of the nutrients in the empty carcasses, the water content was greater in all pairs for the glucose rat ($M = 8.55$, $s = 3.47$, $P < 0.0002$), and the protein content was greater for the glucose rat in seven of the eight pairs ($M = 2.76$, $s = 1.48$, $P = 0.0009$). In respect to fat and energy, the fructose rats contained on the average somewhat more than the glucose rats, but the differences were quite insignificant statistically, since they displayed no consistency in direction among the various

TABLE 4

The results of the comparison of glucose and fructose

RAT NO. AND SEX	SUGAR TESTED	DAYS ON TEST	INITIAL BODY WEIGHT	FINAL EMPTY WEIGHT	GAIN IN WEIGHT	TOTAL FOOD CONSUMED	COMPOSITION OF CARCASS						
							Moisture	Protein	Fat	Energy content	Ash	Calcium	Phosphorus
			gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*calories*	*gm.*	*gm.*	*gm.*
143 ♂	Fructose	50	55	246	191	701	154.1	47.5	37.2	579	8.00	2.24	1.53
144 ♂	Glucose	50	53	258	205	701	159.6	51.1	39.5	641	7.51	2.02	1.42
145 ♀	Fructose	70	51	186	135	746	108.0	35.7	35.1	513	6.72	2.11	1.32
146 ♀	Glucose	70	53	195	142	746	116.7	37.3	34.6	517	7.17	2.11	1.36
147 ♂	Fructose	63	44	225	181	784	143.2	45.5	27.9	500	8.43	2.50	1.61
148 ♂	Glucose	63	45	221	176	784	145.1	45.2	23.9	448	7.98	2.25	1.50
149 ♀	Fructose	78	40	168	128	849	103.7	32.0	26.4	407	6.85	2.06	1.22
150 ♀	Glucose	78	40	172	132	849	112.3	36.1	15.3	342	7.29	2.18	1.37
151 ♀	Fructose	78	48	190	142	969	121.5	36.5	26.0	417	8.23	2.46	1.44
152 ♀	Glucose	78	47	197	150	969	131.1	40.3	16.7	376	8.15	2.40	1.57
153 ♀	Fructose	78	35	166	131	807	107.2	32.1	20.2	352	6.84	1.88	1.28
154 ♀	Glucose	78	35	180	145	807	115.2	36.3	23.0	389	7.30	2.13	1.37
155 ♀	Fructose	78	36	163	127	855	103.6	31.9	19.6	345	7.02	1.87	1.30
156 ♀	Glucose	78	36	174	138	855	115.9	35.1	15.9	335	6.84	1.92	1.25
157 ♂	Fructose	56	38	197	159	684	127.2	39.9	20.6	409	7.50	2.08	1.39
158 ♂	Glucose	56	39	213	174	684	141.0	41.8	20.8	429	7.23	2.04	1.41
Averages	Fructose	68.9	43.4	192.6	149.2	799.4	121.06	37.64	26.62	440.2	7.449	2.150	1.386
	Glucose	68.9	43.5	201.2	157.8	799.4	129.61	40.40	23.71	434.6	7.434	2.131	1.406

pairs of rats. The contents of ash, calcium and phosphorus were almost identical on the average for the glucose and fructose rats.

The percentage composition of the empty carcasses revealed interesting differences between the two groups of rats, as the averages and the statistical analyses summarized in table 5 testify.

On the percentage basis, the carcasses of the fructose rats were definitely, if only slightly, drier and fatter than those of the glucose rats, and less certainly they were richer in calcium and ash and poorer in protein. The average ratios of calcium

TABLE 5

A comparison of the percentage composition of the glucose and fructose rats

RATION	MOISTURE	PROTEIN	FAT	ASH	CALCIUM	PHOS-PHORUS	ENERGY IN CALS./GM.
Fructose	62.80	19.51	13.82	3.90	1.126	0.725	2.282
Glucose	64.46	20.09	11.62	3.74	1.077	0.707	2.144
Statistical analysis of paired differences							
Mean [1]	−1.66	−0.579	+2.20	+0.162	+0.0486	+0.0176	0.139
Standard deviation	1.50	0.755	2.44	0.196	0.0614	0.0479	0.164
Probability	0.011	0.042	0.024	0.049	0.037	0.18	0.030

[1] A + sign indicates a greater average content in the fructose rats. A — sign indicates a greater average content in the glucose rats.

to phosphorus were 1.55 to 1 for the fructose rats and 1.52 to 1 for the glucose rats.

A digestion trial on all pairs of rats in this comparison, revealed a lower digestibility of the fructose ration. In seven of the pairs, the weight of dry feces formed on the same amount of food was greater on the fructose than on the glucose diet, the difference averaging 14.9%. In this case the probability of a chance outcome is 0.036. The weight of nitrogen in the fructose feces was greater in seven pairs than that in the glucose feces, but in this case the average difference is not significant statistically ($P = 0.063$). While the percentage of calcium in the dry feces was significantly greater on the glucose (6.86%) than on the fructose (6.09%) diet, with P

equal to only 0.011, the absolute amount of fecal calcium averaged higher on the fructose diet, although on the average the difference was slight and quite insignificant, P being 0.17. The apparent digestibility of the dietary energy was greater in seven of the eight pairs for the glucose diet, the average coefficients being 92.45 and 93.76. The difference in digestibility, though slight, was highly significant with $P = 0.016$.

In order to detect a possible differential effect of the carbohydrate components of the two experimental diets on the voluntary activity of the rats, all pairs were put into revolving cages equipped with revolution counters for periods of 43 to 56 days. In six of the eight pairs the glucose rat proved to be the more active, although the probability of a fortuitous outcome was so large (0.044) that it can hardly be neglected. Hence, the results of this test merely suggest that the glucose diet induced the greater activity.

Glucose versus lactose. The comparison of glucose and lactose (rations 4 and 5) was not particularly successful, except as it confirmed previous work to the effect that synthetic rations containing as high as 60% of lactose are unphysiological. The lactose rats soon became bloated and diarrheal. Their growth was slow and their appetite was poor. In one of the eight pairs started on test, the lactose rat died after 7 weeks of experimental feeding, during which no appreciable growth occurred. The results of the growth test and of the carcass analyses are presented in table 6.

The greater growth of the glucose rats in body weight and in all constituents of the carcass except calcium is clearly evident from these data. The results are unanimous among all pairs as regards gain in body weight and content of moisture, protein, fat, energy and ash. With respect to phosphorus, six pairs out of seven favor the glucose ration and the probability of a chance result is so small (0.023) that here also it may be concluded that, compared with glucose, lactose impaired dietary utilization. However, in three of the seven pairs, the calcium content in grams was greater for the lactose rat, and although the average was greater for the glucose rats, the difference between the two groups is clearly insignificant.

TABLE 6

The results of the comparison of glucose and lactose

RAT NO. AND SEX	SUGAR TESTED	DAYS ON TEST	INITIAL BODY WEIGHT	FINAL EMPTY WEIGHT	GAIN IN WEIGHT	TOTAL FOOD CONSUMED	COMPOSITION OF CARCASS						
							Moisture	Protein	Fat	Energy content	Ash	Calcium	Phosphorus
			gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*calories*	*gm.*	*gm.*	*gm.*
215 ♀	Lactose	46	45	63	18	202	44.7	11.7	2.46	89	3.13	0.92	0.58
216 ♀	Glucose	46	45	92	47	202	63.3	19.3	5.73	160	3.80	1.06	0.70
217 ♀	Lactose	56	44	92	48	316	64.5	17.7	4.27	153	4.39	1.28	0.81
218 ♀	Glucose	56	44	115	71	316	73.7	22.9	12.98	264	4.52	1.27	0.85
219 ♂	Lactose	60	47	127	80	403	88.0	25.5	6.49	217	5.61	1.59	1.08
220 ♂	Glucose	60	45	163	118	403	109.0	34.3	12.47	326	6.00	1.70	1.09
221 ♀	Lactose	56	43	105	62	333	71.7	21.5	6.47	181	4.83	1.42	0.91
222 ♀	Glucose	56	46	129	83	333	84.7	26.3	12.29	261	4.94	1.37	0.94
223 ♂	Lactose	56	28	86	58	288	60.7	16.6	3.98	137	3.54	0.97	0.67
224 ♂	Glucose	56	28	111	83	288	74.7	22.4	8.78	216	3.98	1.05	0.76
227 ♀	Lactose	54	36	47	11	194	35.5	8.4	2.28	54	2.78	0.86	0.51
228 ♀	Glucose	54	37	69	32	194	47.6	15.4	2.84	108	3.20	0.91	0.60
229 ♀	Lactose	37	73	109	36	241	75.8	21.5	5.82	176	4.77	1.36	0.90
230 ♀	Glucose	37	72	133	61	241	88.6	26.0	13.44	270	4.59	1.30	0.87
Averages	Lactose		45.1	89.9	44.7	282.4	62.99	17.56	4.539	143.9	4.150	1.200	0.780
	Glucose		45.3	116.0	70.7	282.4	77.37	23.80	9.790	229.3	4.433	1.237	0.830

The percentage composition of the carcasses reveals a somewhat different story, as the averages and statistical analyses in table 7 indicate.

On the basis of percentage composition, the lactose rats contained more water, less fat, energy and, less certainly, protein, but definitely more ash, calcium and phosphorus. The percentage of calcium in the lactose rats was far above normal and in one case, rat no. 227, reached the high value of 1.807. The average ratios of calcium to phosphorus were 1.56 to 1 for the lactose rats and 1.49 to 1 for the glucose rats.

TABLE 7

A comparison of the percentage composition of the glucose and lactose rats

RATION	MOISTURE	PROTEIN	FAT	ASH	CALCIUM	PHOSPHORUS	ENERGY IN CALS./GM.
Lactose	70.57	19.30	4.94	4.73	1.380	0.884	1.550
Glucose	66.94	20.65	8.12	3.89	1.085	0.731	1.944
Statistical analysis of paired differences							
Mean[1]	+3.63	−1.35	−3.18	+0.84	+0.295	+0.153	−0.394
Standard deviation	1.50	1.58	2.09	0.19	0.089	0.035	0.107
Probability	0.0005	0.042	0.0049	<0.0001	<0.0001	<0.0001	<0.0001

[1] A + sign indicates a greater average content in the lactose rats. A — sign indicates a greater average content in the glucose rats.

The digestion trial on five pairs of rats in the lactose-glucose comparison revealed a markedly impaired digestibility of the lactose ration. In all pairs the fecal dry matter and fecal energy were greater for the same food intake for the lactose rat. The fecal nitrogen determination was not successful for two of the glucose rats, but in the remaining three pairs, the fecal nitrogen was definitely greater on the lactose ration. On the contrary, the fecal calcium was smaller in amount in all five pairs for the lactose ration; on the percentage basis the dried lactose feces contained an average of only 3.11% calcium, while the dried glucose feces contained 6.14%. Surprisingly, the prevailing diarrheal condition induced by the lactose ration did not increase the fecal wastage of calcium.

The coefficients of apparent digestibility of energy were much larger in each pair for the glucose ration, the averages being 88.03 for the lactose ration and 94.30 for the glucose ration.

DISCUSSION

As compared with glucose, the reference sugar in all three comparisons, sucrose, fructose and lactose, induced greater losses of organic nutrients in the feces, as evidenced by significantly greater excretions of dry matter, nitrogen and energy in the feces for equal intakes of food. The food energy of the sucrose ration was 99.31% as digestible as that of the glucose ration with which it was compared, that of the fructose ration 98.60% as digestible, and that of the lactose ration 93.35% as digestible.

In the glucose-sucrose comparison the differences in digestible nutrients consumed by pair mates brought about by the difference in the carbohydrate components of the diets were so small in magnitude, even though great in statistical significance, that neither the rate of growth nor the total deposition of protein, fat or energy in the carcasses were significantly affected.

In the glucose-fructose comparison, the greater intake of digestible organic nutrients by the glucose rats induced a significantly greater rate of growth which was entirely accounted for by a greater deposition of water and protein in the tissues. From the data of the digestion trial, it may be computed that the glucose rats on the average consumed 496 mg. more of digestible nitrogen than did the fructose rats. Their carcasses contained on the average 440 mg. more nitrogen. The difference in intake of digestible energy, amounting to 42 calories in favor of the glucose rats, equivalent to 0.6 calorie per day, was evidently too small (Mitchell, '35) to induce a greater storage of energy in the carcasses of the glucose rats during the period of experimental feeding.

The greater impairment in the digestion of organic nutrients by lactose than by sucrose or fructose, was associated with much smaller gains in weight by the lactose rats than by

their pair mates on the glucose ration and with distinctly smaller depositions of organic nutrients in their bodies, Consuming 1.52 gm. less digestible nitrogen than their pair mates, the lactose rats deposited 1.01 gm. less nitrogen in their tissues. Consuming 72 calories less of digestible energy, they deposited 85 calories less energy in their carcasses.

While sucrose, fructose and lactose impaired to variable extents the digestibility of the organic nutrients in the experimental rations as compared in each case with glucose, they favored the utilization of calcium and, in the case of lactose, the utilization of phosphorus also. The sucrose rats deposited 7.6% more ash and 11.7% more calcium in their bodies than their pair mates receiving the glucose ration, and these differences were evident in eight of the nine pairs. The mineral content of the feces was not determined in this test.

In the glucose-fructose comparison, neither the deposition of ash, calcium and phosphorus in the bodies of pair mates, nor the excretion of calcium in the feces was significantly different. However, the percentage of calcium, but not of phosphorus, in the carcasses of the fructose rats was significantly higher than that in the carcasses of the glucose rats.

The lactose rats, growing at a rate equal to only 60% of that of their glucose controls, deposited 97% as much calcium and 94% as much phosphorus in their bodies. On an equal empty weight basis, their carcasses contained 28% more calcium and 21% more phosphorus than those of their pair mates. The losses of calcium in the feces were significantly greater on the glucose than on the lactose ration, the difference averaging about 10%.

In the glucose-fructose comparison, and particularly in the comparison of glucose and lactose, the utilization of calcium or of calcium and phosphorus in the non-glucose rats seemed to be limited by the slower growth occasioned by the impairment in the utilization of the organic nutrients. Indicative of this limitation is the greater percentage content of the non-glucose rat in calcium or in calcium and phosphorus both. In these cases, therefore, we may reasonably conclude that if the

intake of digestible organic nutrients had been the same for pair mates, the fructose and the lactose rats would have stored more calcium than their glucose controls, and the lactose rats would have stored more phosphorus.

SUMMARY AND CONCLUSIONS

When sucrose, fructose and lactose constitute 60 to 70% of the diets of growing rats, these diets being otherwise complete, the digestibility of the organic nutrients of the diets, as compared with that of diets containing equal concentrations of glucose, is distinctly impaired, the impairment being least for sucrose, intermediate for fructose, but by far the greatest for lactose.

The slight impairment in the digestibility of the organic nutrients in the sucrose ration did not affect the rate of gain of the rats nor the composition of the gains in such nutrients. On the fructose ration, the rate of growth was definitely slower as was the rate of deposition of protein. But on the lactose ration, the greatly impaired digestibility induced markedly slower rates of growth and rates of deposition of all organic nutrients.

The fecal wastage of calcium was not increased by fructose and lactose as compared with glucose, and the metabolic utilization of calcium was definitely increased by sucrose, fructose and lactose as compared in each case with glucose. Lactose, in addition, definitely promoted the utilization of phosphorus in metabolism as compared with glucose.

It may be concluded, therefore, that the differences in the metabolic disposal of the various sugars studied and their different physiological effects, observed when the sugars are administered alone, largely disappear when they are fed as components of a complete diet. Administered in a complete diet, the nutritive values of the sugars appear to be very much the same except for differential effects on the digestibility of the organic nutrients of the rations. Sucrose, fructose and lactose do seem to favor the utilization of calcium more than does glucose, while lactose promotes also the utilization of

phosphorus.[1] But these effects too may be the result of intestinal conditions more favorable to the absorption of calcium and of phosphorus prevailing on diets containing 60 to 70% of sucrose, fructose and lactose, as compared with those conditions prevailing on a predominantly glucose diet.

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ACTIVATABILITY OF MILK AS AFFECTED BY FEEDING ERGOSTEROL TO COWS

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The absorption by animals of plant sterols, particularly ergosterol, has been studied by several investigators. Schönheimer and his associates ('29, '30) were not able to detect the absorption or storage of ergosterol in mice, rats, dogs, cats or rabbits. Yuasa ('29) demonstrated an increase in the sterol content of the portal blood of dogs following a meal rich in cholesterol, but was unable to show an increase when the plant sterol, sitosterol, was fed. Using laying hens, Schönheimer and Dam ('32) report an increase in the ergosterol content of the yolks of eggs laid by hens which were fed 50 mg. of ergosterol daily. The concentration of ergosterol in the yolk was increased from a normal of 0.0018% to 0.0027%. These studies indicate that the plant sterols are not absorbed by mammals in sufficient amounts to be detected chemically, but they are absorbed by birds in detectable amounts.

It was our purpose to determine whether the absorption of ergosterol could be demonstrated by biological methods and also whether the presence of ergosterol in milk increased its activatability. The latter question is of practical importance since the degree to which milk can be activated is limited, due to the development of an unpleasant taste and odor in the milk by prolonged irradiation.

PROCEDURE

The effect of the direct addition of ergosterol on the activatability of the milk was studied first. Ergosterol dissolved in

a mixture of 1 part chloroform and 4 parts propylene glycol was mixed into 5 gallons of milk to make up batch no. 1. A similar mixture without the added ergosterol constituted batch no. 2. The concentration of added ergosterol in batch no. 1 was 0.42 mg. per quart.

Each batch of milk was irradiated for 75 minutes by recirculation at the rate of 12 gallons per minute through an RUV machine equipped with a high pressure mercury vapor lamp operating at 170 V. Samples of each batch were assayed [1] with the following results:

TABLE 1

Tests of milks irradiated with and without added ergosterol

BATCH	DESCRIPTION	TEST LEVEL (USP UNITS PER QUART)	AVERAGE RESPONSES	
			Assay group	Reference group (2.7 USP units)
I	0.42 mg. added ergosterol per quart	1350	2.4+	2.2+
		2700	1.9+	2.3+
II	No added ergosterol	675	2.3+	2.2+
		1350	1.2+	2.3+

The data indicate that the presence of an added 0.42 mg. of ergosterol per quart of milk resulted in an increased activatability of the milk such that it acquired at least twice the antirachitic potency that ordinary milk attains with a comparable exposure to ultraviolet light. Under these conditions of irradiation the increased vitamin D potency amounted to more than 675 U.S.P. units per quart. A statistical examination of our own assay results, as well as the data given by Bills and associates ('31) and by Coward and Key ('33), indicates that

[1] The assays were made in accordance with the tentative procedure adopted by the A.O.A.C. (J. Assoc. Off. Agr. Chem., vol. 20, p. 79, 1937) using option no. 4. The supplements were mixed with sufficient amounts of the rachitogenic diet 2965 to last 7 or 8 days. The animals were killed on the eleventh day and line test readings made. The responses were rated in accordance with the table given by Bills et al. ('31), i.e., 2+ indicating a narrow continuous line. The reference assay groups received 2.7 units of the international standard. The supplements for the assay groups were calculated as follows:

$$2.7 \text{ (reference group level)} \times \frac{946 \text{ (cc./quart)}}{\text{Test level (assumed U.S.P. units/quart)}}$$

If the test level were 1350 U.S.P./quart then the supplement calculated by the formula above should amount to 1.89 cc. of the milk per rat.

vitamin D bioassays by the line test technic have a P.E. of approximately 15% when the assay groups consist of ten animals. Since our results indicate an increase of at least 100% in the potency attained by milk containing 0.42 mg. of ergosterol per quart over the control milk with comparable exposure, it should be possible by this method to demonstrate the presence of less than 0.2 mg. of ergosterol per quart, or about 1 part in 5,000,000.

In studying the second part of the problem, as to whether ergosterol when fed to cows is absorbed and secreted in the milk, three groups of three cows each, having approximately the same milk and butterfat production, were selected. These three groups were treated as follows:

Group I, control, regular ration.
Group II, regular ration + 4.3 ounces unirradiated yeast daily.
Group III, regular ration + 120 mg. unirradiated ergosterol daily.

After 2 weeks on the foregoing rations the milk production for 1 day was saved for irradiation. A 10-gallon aliquot was used for each group. The irradiation of each lot of milk was carried out in the RUV machine for 120 minutes by re-circulating at the rate of 12 gallons per minute.

These milks were tested at two levels. Each of the three milks was shown to contain more than 540 U.S.P. units of vitamin D per quart.

On retesting the milks at a 675 U.S.P. unit per quart level, the following results were obtained:

TABLE 2

Tests on irradiated milks

GROUP	DESCRIPTION	TEST LEVEL (USP UNITS PER QUART)	AVERAGE RESPONSES		AVERAGE BUTTERFAT CONTENT	AVERAGE MILK PRODUCTION
			Assay group	Reference group		
					%	*pounds*
I	Control	675	2.4+	1.9+	3.8	33.2
II	4.3 oz. yeast per cow per day	675	2.3+	2.0+	3.9	29.7
III	120 mg. ergosterol per cow per day	675	1.5+	2.0+	3.2	32.2

The experiments indicate that feeding 120 mg. of unirradiated ergosterol (either in the form of dry yeast or as crystalline ergosterol dissolved in oil) per cow per day did not increase the activatability of the milk produced.

After the foregoing feeding work was completed we transferred groups II and III from unirradiated material to irradiated yeast and irradiated ergosterol in oil, respectively. In both cases the cows received 325,000 U.S.P. units of vitamin D per cow per day. After these supplements had been fed for 3 weeks, samples of milk from a day's production were obtained for test. Table 3 gives the results of these tests.

TABLE 3

Tests on milks produced by feeding irradiated yeast and irradiated ergosterol

GROUP	SUPPLEMENT	TEST LEVEL (USP UNITS PER QUART)	AVERAGE RESPONSES	
			Assay group	Reference group
II	Irradiated yeast	485	1.6+	2.4+
		400	1.7+	2.0+
III	Irradiated ergosterol in oil	200	1.4+	2.4+
		135	2.0+	2.0+

The results obtained from feeding irradiated yeast and irradiated ergosterol confirm the previous work of Thomas and MacLeod ('31), Hess et al. ('31) and Russell et al. ('34), showing that the vitamin D fed in the form of irradiated yeast is from two to three times more effective in the production of vitamin D milk than the vitamin D fed as irradiated ergosterol in oil.

SUMMARY

The direct addition of ergosterol to milk increased the activatability of the milk. Addition of 0.42 mg. of ergosterol per quart resulted in the milk attaining more than twice the antirachitic potency of normal milk when the two were given comparable exposures to ultraviolet light.

Feeding ergosterol (dissolved in oil or given in yeast) to cows did not result in an increased activatability of the milk on exposure of the milk to ultraviolet light. This result is in

accord with reported chemical studies which indicate that plant sterols are not absorbed by mammals.

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ON THE SURVIVAL OF THE COMPLETELY DEPANCREATIZED DOG [1]

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Since the discovery of insulin, several reports dealing with the survival of the depancreatized dog receiving insulin have appeared. The early reports indicated a failure to effect survival beyond 7 or 8 months (Allan, Bowie, Macleod and Robinson, '24). Later, by the addition of raw pancreas to the diet, Macleod ('30) effected the survival of two dogs for as long as 4 years after excision of the pancreas. A progress report from this laboratory (Chaikoff, '35) showed that with a suitable diet survival for as long as 5 years was effected in two dogs despite the complete absence of raw pancreas from the diet. Recently, however, Dragstedt and his co-workers ('36) have again reported failure in effecting survivals beyond a few months unless raw pancreas or a fraction of this is added to the diet. In view of these differences of experience, a careful study was undertaken in this laboratory of the length of survival of the completely depancreatized dog maintained with insulin and a diet adequate in calories, proteins, salts and vitamins, but containing no pancreas.

EXPERIMENTAL

All dogs to be depancreatized were carefully selected. For several weeks after arrival at the laboratory, they were fed a

[1] Aided by a grant from the Breon Fund of the University of California Medical School, San Francisco. The insulin was generously donated by Eli Lilly and Company. The assistance rendered by the Works Progress Administration is also gratefully acknowledged.

high-calorie, high-vitamin diet, and only those which—besides being normal in all respects— showed a vigorous appetite for the standardized diet that is fed depancreatized dogs in this laboratory were selected for operation.

Following pancreatectomy each animal received 8 units of insulin twice daily, at 8 A.M. and 4 P.M. Just before each injection of insulin, they were fed the following mixture:

	gm.
Raw lean beef	280 [2]
Sucrose	50
Bone ash	7

Vitamin supplements (A and D as cod liver oil; [3] the B complex in the form of a concentrate obtained from rice bran [4]) were added to the diet mixture twice each week, each animal receiving about 25 cc. of cod liver oil and 15 cc. of the rice bran concentrate in that period. This concentrate contained about 50 international units of $B(B_1)$ per cubic centimeter and 10 modified [5] Bourquin-Sherman units of G (flavin) per cubic centimeter. The same concentrate has also been shown to be a good source of both rat and chick antidermatitis factors (Lepkovsky, Jukes and Krause, '36).

The animals were kept in large cages having metal screen bottoms. At no time during the entire stay in the laboratory were they exposed to extremes of temperature.

During the first week or two after pancreatectomy, the animals showed a diminished appetite, but soon regained a vigorous appetite. In order to avoid chronic undernutrition, animals that failed to regain a good appetite within a month or so after pancreatectomy were discarded. In all animals recorded in this study, the diet mixture was completely ingested

[2] This diet was adopted in September, 1935. Previously the animals had received 225 gm. of lean beef and 70 gm. of sucrose, as well as the other constituents listed.

[3] The standardized cod liver oil used in this study was kindly furnished by Mead Johnson and Company.

[4] The rice-bran concentrate was kindly furnished by Vitab Products, Inc., Emeryville, California.

[5] The basal diet for rat assay was modified to supply the rat and chick antidermatitis factors. We are indebted to Mrs. M. K. Dimick of the Vitab Products laboratory for the assays of this material.

TABLE 1

The maintenance of completely depancreatized[1] dogs for periods longer than 1 year with diets containing neither raw pancreas nor choline supplements[2]

DOG	PRE-OPERATIVE WEIGHT	WEIGHT AT END OF PERIOD OF MAINTENANCE	PERIOD OF MAINTENANCE FOLLOWING PANCREA-TECTOMY	REMARKS
	kg.	*kg.*	*years*	
DA♀	9.5	7.5	5.5	Sacrificed while in good condition
DC♀	11.9	10.7	5.1	Sacrificed while in good condition
DB♂	7.3	7.0	4.2	Sacrificed while in good condition
G8♀	9.0	7.1	3.3	Sacrificed while in good condition
DF♀	10.1	9.4	3.1	Met with accidental death. In good condition at time of accident
DD♂	8.3	7.4	2.8	Survival observations on this diet terminated. Animals in good condition at end of this interval
K♂	11.0	6.5	2.7	Died. Bronchopneumonia and acute pyelitis found at autopsy. Refused food for several weeks before death
DE♀	8.1	6.6	2.3	Survival observations on this diet terminated. Animal in good condition at end of this interval
G1♀	8.0	6.7	1.8	Sacrificed while in good condition
G3♀	8.0	4.5	1.8	Died. Emaciated at time of death despite vigorous appetite throughout[3]
G2♀	10.4	8.0	1.7	Died. Infected hydronephrosis with multiple small abscesses in right kidney found at autopsy
DJ♀	8.9	7.2	1.6	Died. Extensive retroperitoneal hemorrhage and cellulitis with involvement of heart found at autopsy
A3♀	9.3	8.8	1.6	Sacrificed while in good condition
DG♀	8.3	6.7	1.5	Died. Acute urinary tract infection found at autopsy. Refused food for 2 weeks prior to death
MF♀	9.6	8.5	1.3	Sacrificed while in good condition
A1♀	8.8	7.3	1.3	Sacrificed while in good condition

[1] Completeness of pancreatectomy confirmed in all cases at necropsy.

[2] It should be noted that the diets employed were not choline-free. The term 'supplements' refers to choline in addition to that already present in the high-calorie, high-protein, high-vitamin diet fed in this laboratory.

[3] The degree of emaciation shown by this dog was not observed in other animals. Such variations might be the result of variations in the degree of absorption to be found after pancreatectomy (McClure, Vincent and Pratt, '17).

within a few minutes after it was served. On occasions a few of the animals showed a temporary loss of appetite, but this rarely lasted longer than 1 or 2 days.

All animals were subjected to a careful search for pancreatic tissue at necropsy.[6] The completeness of all pancreatectomies recorded in tables 1 and 2 was confirmed.

RESULTS

Table 1 records the observations on dogs maintained between 1 and 5.5 years, table 2 those on the dogs that remained in this laboratory for periods less than 1 year.

TABLE 2

Summarized results of completely depancreatized[1] dogs maintained for less than 1 year

PERIOD OF MAINTENANCE AFTER PANCREATECTOMY	NUMBER OF DOGS	SACRIFICED OR TRANSFERRED TO OTHER EXPERIMENTS AT END OF MAINTENANCE PERIOD WHILE IN GOOD CONDITION	NUMBER THAT DIED DURING INTERVAL STUDIED
months			
7–12	7	7	0
6– 6.9	3	3	0
5– 5.9	6	5	1[2]
4– 4.9	12	12	0
3– 3.9	14	13	1[3]

[1] Completeness of pancreatectomy confirmed at necropsy.
[2] Death associated with loss of weight, jaundice and bile in urine.
[3] Did not finish meals completely. Died in hypoglycemia.

Sixteen animals were maintained here for periods between 1.3 and 5.5 years after excision of all pancreatic tissue (table 1). They may be grouped as follows:

1. Eight dogs were maintained for 1.3 to 1.8 years. Four of these were removed from the survival study while in good nutritional state and, to all external appearances, normal. Two of the eight dogs died suddenly; at autopsy one showed a hydronephrosis of the right kidney; in the other, an extensive infection was found in many tissues.

[6] We are indebted to Dr. C. L. Connor and Dr. G. R. Biskind of the Division of Pathology for examination of all tissues in this study.

2. Five dogs survived for periods between 2.3 and 3.3 years. Four of these were in good shape at the time the survival study was terminated. Dog K suddenly refused food and died shortly thereafter.

3. Dogs DA, DB and DC are specially significant in that their periods of survival are the longest so far recorded for completely depancreatized dogs maintained on a diet adequate in all respects but containing no pancreas. All animals were active and possessed a vigorous appetite at the time the survival periods were terminated for examination of their tissues. By external appearance they could not be distinguished from normal dogs.

The forty-two dogs recorded in table 2 were kept in the laboratory for periods less than 1 year and, with the exception of two animals, all were in good shape at the time they were sacrificed.

DISCUSSION

The results of the present study show quite clearly that survival for as long as 4 or 5 years is possible in the completely depancreatized dog so long as he is supplied with insulin and a diet containing large amounts of those constituents known to be essential for the maintenance of a normal dog under optimal conditions, namely proteins, vitamins and salts. In view of the impaired absorption resulting from excision of the gland, the use of a high-calorie, high-protein, high-vitamin diet such as the one used in this study is necessary for prolonged survival. Moreover, since the present purpose was to investigate to the fullest extent possible the effects of the excision of the pancreas without the complications induced by undernutrition, only those animals—with few exceptions—were retained that showed a vigorous appetite for the diet employed.

Since the survival periods recorded here are the longest so far reported, it may be concluded that raw pancreas or other

dietary supplements such as choline [7] are not necessary for the survival of the completely depancreatized dog receiving insulin.

It should be emphasized at this point that despite their survival for these long periods, completely depancreatized dogs manifest certain abnormalities when maintained under the conditions of this study. Indeed, it has been shown in this laboratory that fattiness of the liver is not the only pathological change encountered in the depancreatized dog receiving no raw pancreas but an otherwise adequate diet. The following are among the other changes noted:

1. Cataracts appear as early as 1 year after pancreatectomy (Chaikoff and Lachman, '33).

2. The blood lipid constituents, in particular cholesterol, undergo marked alterations (Chaikoff and Kaplan, '34). These changes may appear as early as 3 weeks after pancreatectomy.

3. Although fatty livers appear early and remain for long periods following excision of the gland, a regression in the fat content of this organ occurs if the animals survive long enough (Kaplan and Chaikoff, '37). This regression is associated with a marked proliferation of fibrous connective tissue arising around the portal triads. In three dogs that survived from 4 to 5.5 years, the livers showed extensive periportal fibrosis with irregular lobulation indicative of cirrhosis.

It may be concluded that in a large number of cases these pathological changes need not interfere with survival so long as the diet is adequate. The alterations in the lipid content of blood and liver observed under these conditions, however,

[7] While it is here shown that choline supplements are not necessary for survival, it should not be inferred that the choline content of the diet is without significance in this regard. The stock diet employed in this laboratory supplied approximately 400 mg. of choline daily. To prove whether choline is an essential constituent for the survival of the completely depancreatized dog treated with insulin would necessitate the use of a diet adequate in all other respects (i.e., calories, proteins, salts and vitamins) but free from choline. As yet no such experiment has been conducted on depancreatized dogs.

are not the result of a deficient supply of insulin, for it has been shown in a series of completely depancreatized dogs recorded elsewhere (Kaplan and Chaikoff, '37) that so long as pancreas is added to the stock diet and its feeding begun immediately after pancreatectomy, the amount of insulin administered, namely 16 units daily, not only keeps the liver lipids at normal levels but also permits the maintenance of a body weight that is either normal (preoperative) or well above the normal.

Although a search at necropsy revealed a complete absence of pancreatic tissue in all animals recorded in this study, the following data are of interest regarding the state of these dogs. The sugar level of the blood and the excretion of glucose in the urine were repeatedly examined during their stay in the laboratory. The following blood sugars taken in the postabsorptive state [8] are representative: Dog DA, 384 mg. per cent; dog DB, 259; dog DC, 272; dog DD, 358; dog DE, 417; dog DF, 476. Glucose, in varying amounts, was always present in the 24-hour sample of urine. When the insulin was decreased or completely withdrawn, an immediate increase in the output of both glucose and nitrogen occurred, and this was followed by a loss of weight and an inability to maintain a positive nitrogen balance.

Previous attempts at survival of the completely depancreatized dog in the absence of raw pancreas and choline supplements.[9] A number of cases have been cited as evidence against lengthy survival, and it has been maintained that supplements either of pancreas (or a fraction thereof) or of choline [9] are necessary for survival beyond a few months. An examination of the diets employed, however, reveals that in some cases they were inadequate in one or more respects and that the importance of feeding a diet high in vitamins and protein to

[8] Blood was taken for analysis between 8 and 9 A.M.; the dogs had received their last injection of insulin and their last meal at 4 P.M. of the previous day. This state of the animal, in which it has been deprived of both food and insulin for 16 hours, is here referred to as the postabsorptive state.

[9] As noted above, 'choline supplements' refer to choline other than that contained in the dietary constituents.

balance the loss of pancreatic enzymes was not always recognized.

1. Allen, Bowie, Macleod and Robinson ('24). The diet employed consisted solely of meat and sucrose. It need not be stressed that this diet is so inadequate in vitamins and salts that early death is by no means surprising.

2. In Fisher's work ('24) no mention is made of the diet employed or of the use of vitamins.

3. Bliss ('22) worked with a single dog that was very weak and emaciated before pancreatectomy. The diet consisted of a ground hash of bread and cooked meat moistened with hot soup; occasionally milk was also given.

4. In the more carefully conducted work of Hershey and Soskin ('31), vitamin deficiencies were recognized and reported. Hershey and Soskin obtained survivals for as long as 11 months without the aid of raw pancreas or lecithin supplements.

5. Penau and Simonnet ('26) report survival of several completely depancreatized dogs for periods over 2 years with diets containing no pancreas. Their animals were fed at frequent intervals a diet containing boiled beef, fresh milk, bread, sugar and bone ash.

6. Dragstedt et al. ('36) state that their completely depancreatized dogs fail to survive more than a few months. In view of the foregoing observations, such a short period of survival is indeed surprising. Each of Dragstedt's animals received daily a diet consisting of 400 gm. of meat, 400 cc. of whole milk and 100 gm. of white bread. All these constituents are poor sources of vitamin $B(B_1)$ (Cowgill, '34; Gunderson and Steenbock, '32; Leong and Harris, '37; Roscoe, '31; Samuels and Koch, '32; Sure, '33). It is possible that the vitamin $B(B_1)$ content of the diet is on the borderline of the minimum requirements for the normal dog. This diet is low, moreover, in vitamin D, and although it may approximate the requirements of this constituent for the adult dog, it is probably not ideal for a growing animal.

Because of the tendency to pathological changes in the liver and other tissues, it is by no means unlikely that an adequate supply of vitamins, proteins and salts is of greater importance in the maintenance of the depancreatized dog than in that of the normal animal. A diet meeting the minimum requirements of a normal dog may be seriously deficient for an animal suffering from impaired absorption. Dragstedt and co-workers ('36) report that their animals suffered from loss of appetite, loss of weight, apathy and muscular weakness. These symptoms are similar to those associated with vitamin B deficiency in the dog, namely anorexia, loss of weight and loss of control of the limbs (Cowgill, '34). It is also interesting to note that convulsive seizures are not uncommon occurrences in dogs subsisting on a diet deficient in vitamin B_1. The importance of providing liberal amounts of vitamins for dogs deprived of pancreatic juice was previously pointed out by Handelsman, Golden and Pratt ('34).

It must now be apparent that in most cases where complete failure in survival was reported due consideration was not given to the vitamin content of the diet. Even with the diet employed in this laboratory, a loss of weight usually occurs after pancreatectomy (compare table 1). It is obvious, therefore, that the caloric intake is not excessive; nor is too much protein given. Indeed, in view of the impaired digestion it is advisable to give large amounts of protein. The importance of avoiding protein undernutrition by providing an adequate protein intake becomes all the more significant from the showing that diets low in choline but high in fat do not produce fatty livers in rats receiving a good amount of protein (Channon and Wilkinson, '35; Best, Grant and Ridout, '36).

While—in view of the impaired absorption—a diet at least adequate in calories, proteins, vitamins and salts is obviously a minimum requirement for survival, the pathological changes that appear in these animals show quite clearly that survival does not occur under optimum conditions. In regard to one of these changes, namely the fatty liver, it has already been shown that this can be prevented or impeded by the addition

of pancreas, either raw or autoclaved, to the diet (Kaplan and Chaikoff, '37). Choline has also been shown to be effective in preventing the deposition of fat in the liver (Best, Ferguson and Hershey, '33). Approximately 400 mg. of choline are provided daily by the stock diet employed in this study. The relation of this amount of choline and of other possible limiting factors to survival and pathological changes is at present under investigation.

SUMMARY

1. When maintained with insulin and a high-calorie, high-protein, high-vitamin diet, completely depancreatized dogs may survive for as long as 4 to 5 years. The pathological changes observed in these animals during survival are recorded.

2. This length of survival makes it unnecessary to assume that raw pancreas or extracts thereof or choline supplements (i.e., in addition to that contained in the dietary constituents) are essential for the survival of the completely depancreatized dog receiving insulin.

3. The previous failures to obtain lengthy survival of completely depancreatized dogs maintained with insulin and a diet containing no pancreas are examined and shown to be of questionable significance in view of the inadequate diets employed.

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animals when placed on the rachitogenic diet. Others have considered the problem from the standpoint of environmental factors such as the temperature and the humidity of the environment. In fact, Tourtellotte and Bacon ('35) have presented data from which they have drawn the conclusion that variations in the sensitivity of the rachitic test animals are due to fluctuations in laboratory temperature during the rachitic and test periods. These investigators intimate that high environmental temperature predisposes to short depletion periods and low biological potencies.

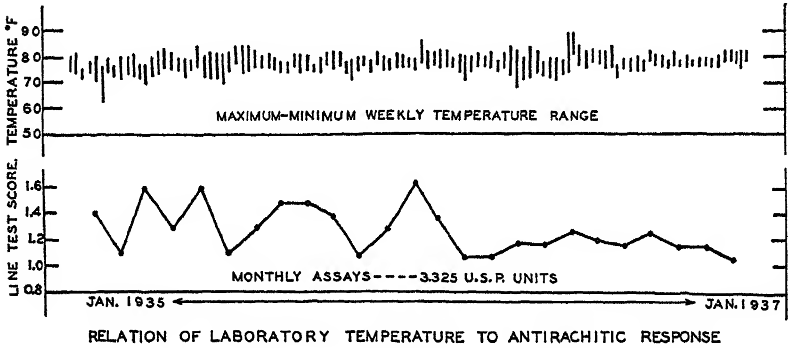

Fig. 1 Showing the relation of the variations in laboratory temperature to the antirachitic potency of the U.S.P. reference cod liver oil as determined by monthly assays during the years of 1935–1936.

If such physical factors as temperature and humidity should be found to be the principal causes of these variations in biological response, this condition might be eliminated once and for all by properly air conditioning the biological laboratory. However, the data available in our laboratory when the above article appeared, failed to show that any uniform relationship existed between the variable sensitivity of our test animals to rickets and the temperature of the experimental laboratory (fig. 1). Realizing, however, that our data had been obtained under a somewhat restricted range of temperatures, we felt that it was not a true index of the influence of temperature on the above condition. It seemed to us that the effect of en-

vironmental temperature on the biological response of rachitic animals could be determined most accurately by conducting simultaneous tests at accurately controlled temperatures, using uniform test animals and maintaining all other factors comparable. The following pages describe such a study.

EXPERIMENTAL

Since most biological laboratories are maintained at temperatures approximating 80°F., we decided to maintain our control animals at this temperature. For the extreme, we chose temperatures approximating 70° and 100°F., which we believed to be sufficiently wide to cover the most practical operating conditions. In order to maintain these temperatures under thermostatic control, a special cabinet was constructed of insulating material around one of our regular three-tier laboratory cage units. The three compartments, each containing one tier of cages, were insulated from each other by a double layer of the insulating material. The surfaces of the compartments were reinforced by wooden framework. In order to facilitate the cleaning and the care of the experimental animals, all compartments were provided with a door on each side. Each compartment, containing eight individual cages, was equipped with heating or cooling elements, thermostatic controls, and other necessary equipment to maintain their temperature at the desired level. For the top and the middle compartments, electrical heating elements were used. The temperature of the bottom compartment was maintained by means of a copper coil through which a regulated amount of cold water flowed. This coil was mounted against the top of the compartment and under it was hung a drip-pan to catch and carry away condensed water. When the necessary equipment had been assembled and put through some preliminary tests, it was found that the copper coil in the bottom compartment was not sufficiently large to reduce the temperature of that compartment to 70°F. under all seasonal conditions. It would, however, maintain a temperature of 73 ± 1°F. Because of this fact, the temperature of the

middle compartment was adjusted to 84° instead of 80° as previously planned. The experimental equipment, therefore, when placed in operation consisted of three compartments, each of which contained eight individually caged animals that were maintained at temperatures of approximately (±1°F.) 73, 84 and 100°F., respectively.

This investigation consisted of two parts: first, to determine the effect of temperature on the production of rickets, and secondly, to determine the effect of temperature on the recalcification of rachitic rats when fed a definite unitage of vitamin D. In the first of these studies, two series of animals were used, while in the second phase four regular series were found to be necessary and, in addition, several control groups. All test animals were of the same strain, purchased from the same source and were of approximately the same age when placed on experiment. In placing the animals on experiment, unusual care was exercised to select only animals of the same sex and of the same weight for the three compartments. All animals were fed liberal quantities of the rachitogenic diet (Steenbock and Black no. 2965, '25) and were supplied with clean distilled water at all times. In fact, the care and handling was the same as that accorded our regular vitamin D assay animals. Animals used in the recalcification studies were given 2 U.S.P. units of vitamin D on the first and again on the fourth day of the 10-day curative period. The temperatures of the three compartments were carefully recorded each day at a regular hour. The right hind leg of each animal was x-rayed on the eighteenth and the twenty-first (last) day of the depletion period and again on the seventh and the tenth (last) day of the curative period. At the end of the experiments all animals were anesthetized with ether, the blood removed by heart puncture, the tibiae removed for bone ash determinations, and the femora for 'line tests.' The inorganic calcium and the inorganic phosphorus of the serum of the combined blood from all animals in each compartment was determined by the Clark-Collip ('25) modification of the Kramer-Tisdall ('21) method and by the

Youngburg et al. ('30) method. The flesh-free tibiae were dried, extracted in a Soxhlet extractor with ethyl alcohol for a period of 12 hours and again extracted for a like period with sulfuric ether. The extracted bones were again dried, weighed and ashed at a red heat until constant weight resulted.

DATA

The data obtained in these studies have been condensed in tabular form and are presented in the following table. Owing to the close similarity between the x-ray findings among the groups of each series, it was found impossible to express the stages of calcification in terms of a quantitative measure; therefore, no data obtained by this criterion are included. The x-rays were of considerable value, however, in following the progress of decalcification during the depletion period. The relative degrees of decalcification manifested by the negative control animals at the end of the depletion period were evaluated in terms of an arbitrary scale in which conditions approximating mild, moderate and severe rickets were expressed as — 1, — 2 and — 3, respectively. The degrees of recalcification as indicated by the 'line test' were evaluated in the usual manner in which a value of 2 represented a thin but continuous line of darkened granules appearing on the metaphyseal side of the epiphyseal cartilage. Greater or lesser degrees of recalcification were designated accordingly. The following data represent the average values of all animals of the respective groups.

DISCUSSION

Since the above experiments were carried out during a period extending from late winter to early fall, the test animals used should have been fairly representative of those commonly employed in biological assays so far as the seasons of the year are concerned. With every experimental series, unusual efforts were made to select only vigorous animals of the same sex and of the same weight for each of

TABLE 1

Showing the average values obtained from the various groups of animals during the investigation. These values have been tabulated in the order of increased environmental temperature and also according to the three stages of calcification represented

GROUP NO.	NUMBER OF ANIMALS	AVERAGE TEMPERATURE OF COMPARTMENT	AVERAGE INITIAL WEIGHT	DEPLETION PERIOD	CURATIVE PERIOD	UNITS VITAMIN D FED	AVERAGE FINAL WEIGHT	AVERAGE DAILY FOOD INTAKE	AVERAGE 'LINE TEST' SCORE	SERUM Ca per 100 ml.	SERUM P per 100 ml.	AVERAGE BONE ASH
Negative controls—rachitogenic diet without supplement												
		°F.	gm.	days	days	U.S.P.	gm.	gm.	– or +	mg.	mg.	%
1-A	8	74	45	21	0	0	65	5.7	—1.9	10.4	3.3	36.6
2-A	6	72	45	21	0	0	61	5.1	—1.8	11.1	4.5	36.7
1-B	8	84	45	21	0	0	62	5.0	—1.8	12.3	3.5	38.8
2-B	8	84	44	21	0	0	60	4.6	—2.0	11.3	4.3	38.4
7-B	8	84	43	31	0	0	70	4.9	—2.5	10.1	2.9	29.8
1-C	7	101	45	21	0	0	58	3.6	—2.0	10.8	3.2	37.7
2-C	4	100	43	21	0	0	56	3.4	—2.0	12.3	4.6	40.3
Experimental—rachitogenic diet plus vitamin D supplement												
3-A	5	73	43	21	10	4	67	6.5	+1.6	9.5	6.3	32.8
4-A	8	72	45	21	10	4	67	6.3	+1.5	10.7	9.2	43.5
5-A	7	73	46	21	10	4	70	5.8	+1.9	10.7	5.2	34.3
6-A[1]	6	72	44	21	10	4	64	5.9	+1.8	12.7	7.7	36.4
3-B	8	84	45	21	10	4	66	5.6	+1.3	12.6	6.7	34.5
4-B	7	85	45	21	10	4	68	5.5	+1.3	10.8	8.4	41.2
5-B	8	84	46	21	10	4	72	5.0	+1.9	12.8	4.9	32.7
6-B[1]	8	84	44	21	10	4	61	4.7	+1.5	12.5	5.0	36.0
3-C	(All animals over heated)											
4-C	7	100	45	21	10	4	66	3.9	+1.7	10.5	6.8	43.6
5-C	6	101	46	21	10	4	61	3.1	+1.2	11.7	4.9	34.7
6-C[1]	6	100	44	21	10	4	56	3.2	+1.7	12.4	6.2	36.7
Positive controls—receiving breeding colony diet												
8-B	8	84	44	2 days at 84°F.			48	6.9	Normal	11.3	10.1	51.7
9-B	6	84	43	6 days at 84°F.			60	8.2	Normal	11.7	9.3	52.2
10-B	8	84	45	10 days at 84°F.			71	9.6	Normal	11.4	9.4	53.5

[1] All animals composing these groups were females. All other animals used in the investigation were males.

the three compartments. In fact, the method of selection was so exacting that in some series the individual weights of all animals of the three compartments, when placed on the rachitogenic diet, did not differ by more than 3 gm. All animals used were males except those of the sixth series, which were all females.

For the sake of reference, the three experimental compartments were designated from bottom to top as compartments A, B and C, respectively. As previously stated, the approximate temperatures at which these compartments were maintained were 73, 84 and 100°F., respectively. The various series of animals used were designated numerically in chronological order. A reference to a particular group, for example group 5-B, denotes, therefore, the fifth group of animals maintained in compartment B at a temperature approximating 84°F. Other groups will be similarly designated.

The relation of environmental temperature to the daily food intake of the various test animals became distinctly evident during the first series of studies and remained equally evident throughout the entire investigation. As was to be expected, those animals which were subjected to the lower temperatures consumed greater quantities of the rachitogenic diet. In fact, at the end of the depletion period those groups of animals which were maintained at temperatures of 84 and 73° had consumed, on an average, 13 and 31% more food, respectively, than had similar animals maintained at 100°F. When the experiments were continued over a 10-day curative period, these differences in food consumption increased to 50 and 82%, respectively. The average increase in body weight was also greater at the lower temperatures. This would indicate that, under the conditions of the investigation, the rat's requirement for food was not restricted to that required for energy production plus the additional amount required to produce a uniform rate of growth. The relative amounts of food required to produce a unit gain in body weight at the temperatures studied were: 5.9, 5.1 and

4.5 gm., respectively, at the end of the depletion period and 8.7, 7.5 and 6.6 gm., respectively, at the end of the curative period.

The degree of rickets manifested at the end of the depletion period, as indicated by the line test, showed no significant differences that might be in any way attributed to differences in environmental temperature. There was a very slight indication that more severe rickets were produced at the higher temperature, but it appears doubtful if the method of scoring the rachitic condition was sufficiently sensitive to make such differences significant.

The line test score as determined at the end of the curative period did not indicate that environmental temperature is an important consideration in recalcification in the rat. The average values of the scores do indicate slightly greater recalcification at the lower temperatures, but differences among these values are less than differences among line test values obtained from different groups of animals maintained at the same temperature and otherwise treated identically.

Those animals which had received the rachitogenic diet during a 21-day depletion period likewise failed to show that environmental temperature had any definite effect on serum calcium, serum phosphorus or bone ash. Here again groups of animals of different series which had been maintained at the same environmental temperature showed as much variation in these values as did groups of animals of the same series which had been maintained at different temperatures. At the end of the curative period, however, there were indications that a higher environmental temperature resulted in a higher serum calcium and a lower serum phosphorus. Here again group differences were marked and it appears doubtful if the apparent temperature effects were significant.

A point which appears worthy of mention concerns the relative mortality among the groups of animals maintained at the different temperatures. It may also be well to state that the third group of animals (group 3-C) maintained at the higher temperature was accidentally subjected to an increased tem-

perature (108°F.) during one night of the second week of the experiment, due to the improper functioning of the thermostat. As a result of this increased temperature, a number of the animals died during the night and others were so severely weakened that the entire group was discarded. Other than this group, the total mortalities numbered a total of twenty-seven, of which eight occurred at the lowest temperature, one at the medium temperature and eighteen at the highest temperature. Nothing was observed, however, that would indicate that the higher mortality was in any way related to a more severe rachitic condition.

We were somewhat surprised to find that those animals which had been maintained at the lowest temperature and which had consumed the greatest amount of food, and in consequence had made the greatest gain in body weight, did not show a significant difference in the degree of rickets manifested. In our regular vitamin D assays, where all animals are maintained at a comparable temperature, those animals which make the greatest increase in body weight invariably show the highest degree of rickets.

A general consideration of these data leads us to conclude that environmental temperatures, within the ranges studied, are rather unimportant considerations in either the production or the cure of rickets in the rat. On the other hand, the data do indicate that the history of the test animal previous to being placed on the rachitogenic diet is a worth while consideration. This is well illustrated by the animals comprising the fourth series (groups 4-A, 4-B and 4-C) which showed considerably higher serum phosphorus and bone ash values at all temperatures than did the animals of any of the other series. Such variations in test animals appear to be independent of the body weight when placed on experiment. It is highly probable that this can be at least partially attributed to unrecognized variations in the breeding colony diet.

SUMMARY

A series of studies have been made concerning the effect of environmental temperature on the production and on the cure of rickets in the rat. Comparable experiments were carried out at temperatures of 73, 84 and 100°F., using a uniform rachitogenic diet and carefully selected test animals. The stages of calcification were measured by means of x-rays, line test, bone ash, and serum calcium and phosphorus.

The data obtained in these studies indicate that environmental temperature is not an important consideration in either the production or the cure of rickets in the rat. In fact, undetectable differences in different groups of rats when placed on the rachitogenic diet led to greater variations in experimental results than did a variation of approximately 28°F. in environmental temperature.

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FURTHER STUDIES ON THE EFFECT OF EXCESSIVE VITAMIN A ON THE OESTROUS CYCLE OF THE RAT

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Baumann and Steenbock ('32), Mason and Ellison ('35) showed the effect of avitaminosis A; Sherwood, Brend and Roper ('36) showed the effect of hypervitaminosis A on the vaginal smear of rats. It was therefore deemed advisable in the present investigation to study the effect of excessive vitamin A administration upon the oestrous cycle. A study of the vaginal smear, the uterine and ovarian picture was made to show this phenomenon. The procedure of this investigation was designed to show if possible, the mechanism responsible for these changes.

EXPERIMENTAL

Vaginal smears were obtained on a group of twenty-six rats approximately 150 days of age. From four to thirteen oestrous cycles were observed for the purpose of determining the length of the normal cycle in these animals. Fifteen of the rats were then injected daily with 1500 international units of carotene [1] for a period of 5 to 15 days. Eleven animals were fed 1500 international units of carotene daily for a period of 5 to 32 days. The smears were taken on all animals during the period of vitamin A administration.

Five of the animals were killed after the carotene had been administered 5 to 10 days. Sixteen animals were killed at

[1] The carotene used in the present investigation was generously furnished by S.M.A. Corporation, Cleveland. Ohio.

intervals after carotene had been administered 11 to 32 days. Five rats were allowed to return to the normal oestrous cycle following carotene administration.

In order to prevent a cyclic disturbance due to an upset in nutrition and environment, the animals were kept in a room of constant temperature and given a well-balanced stock diet (Purina chow).

TABLE 1

The effect of carotene injections upon the vaginal smear, uterus and ovary

RAT NO.	NORMAL CYCLES	AVERAGE CYCLE	DAYS OF THERAPY	ABNORMAL	UTERUS	OVARY	ANIMAL KILLED
		days		*days*			
1	10	4.4	5	3	Proestrus 3.0 mm.	Follicles med. and large	5th day
2	13	4.2	7	5	Proestrus 2.3 mm.	Follicles med. and C.L.	7th day
3	13	4.1	10	8	Proestrus 2.7 mm.	Follicles large	10th day
4	8	4.6	10	8	Dioestrus 2.2 mm.	C.L. large	10th day
5	11	4.5	12	10	Dioestrus 2.0 mm.	C.L. large	12th day
6	12	4.2	12	10	Proestrus 2.7 mm.	Follicles small C.L.	12th day
7	12	4.3	15	16	Proestrus 2.9 mm.	Follicles small C.L.	18th day
8	8	4.1	15	16	Proestrus 2.7 mm.	Follicles small C.L.	18th day
9	7	3.9	15	16	Dioestrus 1.7 mm.	Follicles small C.L.	18th day
10	12	4.4	15	15	Proestrus 3.1 mm.	Follicles large C.L.	18th day
11	7	4.2	15	15			18th day
12	7	4.5	15	15	Dioestrus 1.7 mm.	Follicles vary C.L.	18th day
13	8	4.3	15	36			Returned to normal
14	6	4.4	15	33			Returned to normal
15	6	4.4	15	33			Returned to normal

RESULTS

The control animals that had received cottonseed oil showed no upset in the oestrous cycle. The animals that had received carotene continued to show a vaginal smear picture of leucocytes and nucleated epithelial cells during the period of carotene therapy. The injected rats rarely showed cornified cells and the fed animals presented slightly greater numbers of cornified cells in the vaginal smear. In no case were the cornified cells typical of oestrus.

TABLE 2

The effect of carotene feeding upon the vaginal smear, uterus and ovary

RAT NO.	NORMAL CYCLES	AVERAGE CYCLE	DAYS OF THERAPY	ABNORMAL	UTERUS	OVARY	ANIMAL KILLED
		days		*days*			
16	10	4.7	5	3	Proestrus 2.5 mm.	Follicles small C.L.	5th day
17	6	3.8	11	8	Dioestrus 2.1 mm.	Follicles small C.L.	11th day
18	4	4.7	11	9	Dioestrus 2.5 mm.	Follicles vary C.L.	11th day
19	6	4.1	13	11	Proestrus 5.0 mm.	Follicles large C.L.	13th day
20	6	4.1	15	12	Proestrus 4.0 mm.		15th day
21	6	4.1	29	27	Dioestrus 1.7 mm.	Follicles vary C.L.	29th day
22	4	4.7	29	27	Oestrus 6.0 mm.	Follicles large C.L.	29th day
23	5	4.7	29	27	Proestrus 4.0 mm.	Follicles med. and C.L.	29th day
24	6	3.8	32	30	Dioestrus 1.7 mm.	Follicles small C.L.	32nd day
25	8	4.5	15	34			Returned to normal
26	4	5.2	15	17			Returned to normal

The average length of the oestrous cycle was determined from the control series obtained preceding the carotene administration. Vaginal smear records were obtained approximately 10 minutes prior to the death of each animal.

These smears were compared with the expected normal smears that should have been obtained had the animals not received carotene.

Table 3 presents the data obtained from the vaginal smear studies. Thirty to 80 hours would have been required for the animals to reach the calculated stage as determined from the control series. Some variation existed in the length of time for the various cycles in the control series. However,

TABLE 3

The effect of vitamin A upon the oestrous cycle of the albino rat

RAT NO.	AVERAGE CYCLE	VAGINAL SMEAR			CAROTENE THERAPY	SMEAR VARIED
		Beginning of injection	Day killed	Expected smear		
			Carotene injected			
	days				*days*	*hours*
1	4.4	Cor++[1] L+[1]	L+ Ne++[1]	Cor++ L+	5	61
2	4.2	Cor+++	Ne++ Cor+	L+++	7	45
3	4.1	L+ Ne++	Ne++ Cor+	Cor++ L+	10	35
4	4.6	Cor++ L+	L+ Ne++	Cor++ L+	10	55
5	4.5	L+ Ne++	L+ Ne++	L+++	12	40
6	4.2	L+ Ne++	L+ Ne++	L+++	12	30
7	4.3	L++ Ne+	L++ Ne+	L++ Ne+	17	00
8	4.1	L+ Ne++	L++ Ne+	Ne++ Cor+	17	80
			Carotene fed			
20	4.1	Cor+ L++	Ne+++	L+++	13	55
21	4.1	L++ Ne+	L++ Ne+	Cor+++	28	70
23	4.7	Cor+ L++	Ne++ Cor+	Cor++ L+	28	50
24	3.8	L++ Ne+	L++ Ne+	Ne++ Cor+	31	37

[1] The abbreviations are to designate the presence of cornified, leucocytes and nucleated epithelial cells and the plus sign indicates the cell type in the majority.

normal oestrous cycles were never observed during the carotene administration. After the second day of carotene therapy, all animals showed great numbers of nucleated epithelial cells and leucocytes. The majority of animals gave no indication of going through the cornified cell stage. Several animals were not sacrificed and showed normal cycles approximately 20 days following the cessation of carotene administration.

In the study of the uteri as shown in tables 1 and 2, it was found that only five of the twenty-six animals demonstrated

uteri above 3.0 mm. in diameter, the majority of them being apparently in late dioestrus and early proestrus. The ovaries presented a similar picture. There were few large follicles and many corpora lutea in various stages.

DISCUSSION

Since Baumann and Steenbock ('32) reported that excessive cornification, in the vaginal epithelium of the albino rat, was an early manifestation of vitamin A deficiency, and were able to produce dioestrous smears within 1 week and oestrus in 2 weeks after the administration of carotene, it was thought advisable to study the effect of excessive vitamin A since the vitamin has a decided effect upon epithelial cells.

Mason and Ellison ('35) reported a marked increase in cornified cells in avitaminosis A. This effect may have been an indication that the epithelial cells died early and that the body was not able to remove the cornified cells as rapidly as produced. One might have expected therefore a normal or even an excessive amount of vitamin A to have kept these cells in a healthy condition and have prevented cornification.

The excessive vitamin A administration in the present investigation apparently produced an upset in the oestrous cycle as shown from the study of the uteri and ovaries as well as the vaginal smears. Since the control rats gave no indication of a changed vaginal smear picture, it would seem possible that the vitamin A was responsible for an upset of the oestrous cycle, or brought about marked mitosis, thus producing greater numbers of nucleated epithelial cells.

The animals that had received carotene by subcutaneous injection may have shown a more marked effect due to the fact that the vitamin was more efficiently utilized because of a slow absorption and no loss through the alimentary canal.

Further studies on the changed uterine picture are being made for the purpose of checking more adequately the vitamin A effect. The possibility of a hormone-vitamin relationship is also being investigated.

SUMMARY

Vitamin A given in the form of carotene orally and subcutaneously produced a change in the oestrous cycle of the albino rat. The vaginal smears showed nucleated epithelial cells and leucocytes during the entire period of carotene therapy. Very few cornified cells were observed during the experimental period.

Vitamin A given subcutaneously produced a greater effect upon the vaginal smear and uterine pictures than did oral administration.

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THE RESPIRATORY METABOLISM OF RATS RECEIVING A DIET DEFICIENT IN INORGANIC CONSTITUENTS. THE CHANGE IN BASAL METABOLISM [1]

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ONE FIGURE

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The field of respiratory metabolism in relation to mineral deficiencies is largely unexplored, although considerable knowledge is available regarding many effects of such deficiencies which are indicative of respiratory metabolic disturbances. Aside from some rather conflicting reports on the influence of calcium and phosphorus deficiencies there are no data on record, insofar as the writers are aware, which are directly concerned with the respiratory metabolism in relation to mineral deficiencies.

In an investigation of the influence of calcium deficiency on the basal metabolism of rats, Pedotti ('21) observed that after feeding a calcium deficient diet to young rats for a period of 3 weeks the fasting metabolic rate of the subjects was considerably decreased as compared with the fasting metabolism determined prior to this dietary treatment, although the irrita-

[1] The data reported in this paper were taken from a dissertation presented by Max Kriss in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Yale University, 1936.

A preliminary report was presented before the division of biological chemistry, American Chemical Society, eighty-ninth meeting, New York, April, 1935.

[2] Block Fellow in Physiological Chemistry, Yale University, 1934–1935.

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bility of the rats was increased. Subsequent feeding of a diet adequate in calcium resulted in a slight increase in the fasting metabolism, but failed to bring it to the pre-experimental level. The author concluded that calcium deficiency has the effect of lowering the basal metabolism. The possible influences of change in age and of the plane of preliminary feeding on the basal metabolic rate were not considered. The respiratory measurements were made at temperatures close to 20°C., which is much below the critical temperature for the rat.

Riddell, Hughes and Fitch ('33) measured the oxygen consumption of lactating cows kept alternately at normal and at low phosphorus intake. The measurements were made by means of a portable metabolism apparatus in 6-minute periods 12 hours after feeding. The authors observed that the phosphorus deficiency resulted in an increased oxygen consumption indicating an increased rate of metabolism. Kleiber, Goss and Guilbert ('36) determined the respiratory exchange of phosphorus-deficient beef heifers in the fasting condition and also after the ingestion of food. They found that the phosphorus deficiency had no effect on the fasting katabolism, but that it decreased the efficiency of energy utilization.

Observations on the influence of sodium deficiency on the utilization of energy and protein, which have an important bearing on the general subject of the present investigation, were reported by Kahlenberg, Black and Forbes ('37). In a paired-feeding experiment, rats receiving a diet deficient in sodium, stored less energy as fat and protein in their bodies and produced more heat, as compared with rats receiving an adequate supply of sodium. The heat loss was calculated from the analyses of the food, excreta and the rats' bodies.

A possible relationship between mineral deficiency and respiratory metabolism is suggested by the observations on the metabolic, hemopoietic and structural changes resulting from the strict limitation of inorganic salts in the diet (Swanson and Smith, '32, '34; Brooke and Smith, '33; Clarke, Bassin and Smith, '36; Smith and Smith, '34 a; '34 b; Eppright and Smith, '37 a, '37 b). Smith and Smith ('34 a) made the ob-

servation that although rats receiving a low-salt diet gained less in weight in comparison with their calorie controls, their voluntary activity and insensible weight loss, which normally are an index of metabolic intensity, decreased rather than increased. These findings raised a question as to the compensatory mechanism whereby the mineral deficient animal disposes of its available energy supply in bringing about the apparently lowered economy of food utilization. The following possibilities suggested themselves:

The mineral deficiency might have the effect of increasing the basal energy requirements of the animal; if the basal metabolism were unaffected, the specific dynamic effect of the mineral deficient diet might be so altered as to cause an increase in the total metabolism; the processes of digestion and absorption might be disturbed to such an extent as to lower the economy of food utilization; the mode of heat disposal might be so altered by the lack of mineral salts in the diet as to affect the relationship between the metabolism and the insensible weight loss.

The present paper is a record of experiments designed to elucidate the influence of the low-salt diet on the basal metabolism.

EXPERIMENTAL

Twenty male albino rats of the Connecticut Agricultural Experiment Station strain were used in this investigation. Only those were selected which weighed 40 gm. or more at weaning (21 days). This prerequisite was arbitrarily imposed with a view of securing a more uniform rate of growth during the period immediately following weaning, which is designated as the pre-experimental period. During this period the rats were fed an adequate stock diet ad libitum and housed in individual metal cages.

The pre-experimental feeding periods were of 12 to 17 days' duration, terminating when the rats had attained a weight of approximately 120 gm. Food was then withdrawn for 24 hours, after which the rats were subjected to an 8-hour meta-

bolism test. The chief purpose of this initial test was to secure a base line from which to view the results of the metabolic studies conducted after the imposition of the experimental dietary regime.

After the completion of the first basal metabolism test the rats were placed on purified diets by pairs. One rat of each pair received a ration which, exclusive of vitamin supplements, was extremely low in inorganic salts; it was composed of specially washed casein, 18%, dextrin, 55%, and hydrogenated fat, 27%. This diet is designated diet I, or the low-salt diet, and provided 0.075% total ash. Taking into account the voluntarily restricted food intake of the animals consuming the salt-poor ration, Smith and Smith ('34 b) calculated that the following amounts of the inorganic constituents per rat per day were eaten: phosphorus 14.6 mg., total base 0.320 m.eq., calcium 0.013 m.eq., magnesium 0.018 m.eq., potassium 0.228 m.eq., sodium 0.044 m.eq., chloride 0.072 m.eq. The other rat of the pair, a litter-mate, which served as the control, received in addition to diet I, 4% of a satisfactory salt mixture (Osborne and Mendel, '19). This ration containing the mineral salts is designated diet II. All rats received the same vitamin supplements. These consisted of dried yeast (200 mg. per day), alcoholic extract of wheat germ (1 cc. per day, equivalent to 2 gm. of wheat embryo) and 6 drops of cod liver oil per day. Food was offered twice daily in two equal portions, the feeding being controlled by the paired-feeding technique.

The rats were kept on the experimental rations for a period of approximately 3 months. Some of the rats were subjected to basal metabolism measurements at approximately monthly intervals, but all rats which survived were subjected to a final basal metabolism test at the end of the experimental feeding period. All experimental work was conducted, as far as it was possible, with both rats of each pair simultaneously.

The basal metabolism measurements started 24 hours after food and, with very few exceptions, continued for 8 consecutive hours. Measurements of carbon dioxide production were

made at hourly intervals, while determinations of oxygen consumption and of respiratory quotients were made for the entire experimental periods.

The respiration measurements were made by means of an open-circuit respiration apparatus similar in construction to that recently described by Forbes, Kriss and Miller ('34). Certain modifications were introduced, of which the most important were the use of a constant temperature air bath instead of a water bath, the provision for the visibility of the rats at all times by means of reflecting mirrors, and the abandonment of the wire screen cylinder as a means of restricting the movement of the animal. Bright illumination alone sufficed to accomplish the latter purpose.

The respiration chamber consisted of a 1-quart fruit jar 8 inches high and 4 inches in diameter, provided with a screw lid of standard size through which two pieces of copper tubing were fitted which served for connection to the air circuit. An ordinary jar rubber gasket was used to make the connection between the lid and the jar air-tight. Inside the jar was placed a strip of ½-inch galvanized wire screen about 2½ inches in width which served as a platform for the rat, thus preventing it from coming in contact with any excreta voided during the experiment. This screen was held in place by a loop of steel wire which was soldered to it. The size of the jar and the wire screen in relation to the size of the animals used were such that the animals could move about without difficulty.

Uniform conditions of temperature, ventilation and light were maintained throughout these experiments. The temperature of the air bath into which the chambers were placed was maintained close to 28°C. and the rate of ventilation was approximately 60 liters per hour. For illumination one 15-watt electric bulb was placed in the center of the lid of the air bath above each of the two respiration chambers. Under these conditions the activity of the rats was greatly restricted without any signs of discomfort. Close observations of the activity of the animals while in the respiration chamber were useful in reducing the respiration measurements to a basis of comparable inactivity.

The schedule of the respiration experiments is presented in table 1.

TABLE 1

Schedule of experiments

RAT NOS. Low salt / Control	AGE	DAYS ON DIET	RAT NOS. Low salt / Control	AGE	DAYS ON DIET	RAT NOS. Low salt / Control	AGE	DAYS ON DIET
1 and 2	35	..	11 and 12	129	91 and 92	23 and 24	36	..
..[1] 2	91	56	13 and 14	32	..	23 and 24	67	31
3 and 4	36	..	..[2] 14	119	87	23 and 24	93	57
3 and 4	123	87	15 and 16	34	..	23 and 24	119	83
6 and 7	34	..	15 and 16	109	75	25 and 26	39	..
6 and 7	123	89	15 and 16	118	84	25 and 26	68	29
9 and 12	37	..	19 and 20	33	..	25 and 26	94	55
11 and 10	38	..	19 ..[3]	118	85	25 and 26	122	83
9 and 10	125	88 and 87						

[1] Rat 1 died 12/21/34 as a result of salt deficiency.
[2] Rat 13 died 2/9/35 as a result of salt deficiency.
[3] Rat 20 died 2/28/35 as a result of an accident.

RESULTS

The influence of activity

The measurements of the carbon dioxide production at hourly intervals, instead of for the total experimental period, together with the direct observations of the animal, have yielded important information regarding the fluctuations of the metabolism as affected by activity. The carbon dioxide production during the first hour was invariably high. This was associated with the activity which the rat exhibited during the early part of the first hour. It took in most cases from 3 to 5 minutes to weigh the animal with the jar tightly closed. During this time the rat was never entirely quiet. It also required some time for the rat to become adjusted to the environmental conditions after the respiration chamber was connected with the air circuit. After the first or second hour, however, the hourly CO_2 production was relatively uniform. In all cases the subject was found inactive during several consecutive hours. The number of hours during which the rat exhibited no activity varied in different periods. In some cases as many as six or even seven of the eight hourly periods of observation represented periods of inactivity.

The desirability of eliminating effects of activity of the subject from determinations of basal metabolism is obvious. To this end, in our final computations, we have excluded from the average, those results obtained during hours of activity. It was of considerable interest, however, to know to what extent the activity actually influenced the metabolism under our experimental conditions, and to determine what effect, if any, the mineral deficiency had in this respect. For this purpose, we have calculated the average hourly heat production of all the rats in all periods on two bases: 1) including the hours of activity and 2) excluding the hours of activity. The results obtained in the final fasting periods, following the 83 to 92 days of the low-salt feeding, are compared in table 2.

TABLE 2

The average hourly heat production during the final series of experiments, including and excluding hours of activity

RAT NO.	DIET NO.	AVERAGE HOURLY HEAT PRODUCTION		NUMBER OF HOURS AVERAGED IN (2)	DIFFERENCE $\frac{(1)-(2)}{(2)}$
		Including hours of activity (1)	Excluding hours of activity (2)		
		cal.	*cal.*		%
3	I	820	760	5	7.9
4	II	729	659	6	10.6
6	I	757	743	7	1.9
7	II	726	683	6	6.3
9	I	1008[1]	821	4	
10	II	798	736	5	8.4
11	I	836	748	4	11.8
12	II	812	696	3	16.7
14	II	869	757	4	14.8
15	I	718	677	5	6.1
16	II	787	689	5	14.2
19	I	784	740	5	5.9
23	I	775	723	5	7.2
24	II	783	622	3	25.9
25	I	776	700	4	10.9
26	II	695	631	5	10.1
Average	I (low salt)	781	739		7.4
Average	II (control)	774	684		13.4

[1] This high value is due to an abnormal condition of the animal and was not included in the average.

In the initial fasting periods, that is, before the rats were subjected to the purified diets, the differences between the average hourly heat production as calculated on the two bases ranged from 1.8% (with rat 2) to 9.4% (with rat 25), the average difference of all twenty comparisons being 5.4%. In this series of experiments the number of hours which represented periods of inactivity ranged from 4 to 7. In the majority of cases these periods of inactivity were represented by 5 or 6 hours. The same was true in the intermediate fasting experiments. In the latter the increases in the average hourly heat production resulting from the inclusion of the hours of activity were, with two exceptions, less than 10%. The average increase in metabolism with the low-salt animals (5.5%) was, however, considerably less than the average increase (11.4%) with the controls.

In the final fasting periods after 83 to 92 days on the salt-poor diet (table 2), the activity appears to have exerted a considerably larger effect than in either the initial or intermediate periods. Here the differences ranged from 1.9% (with rat 6) to as high as 25.9% (with rat 24). With one exception (rat 25) the differences in heat production due to activity were much less with the low-salt animal than with the control. The average difference for the low-salt animals was 7.4%, as compared with 13.4% for the controls. Indeed, it was observed that the low-salt animals were less active and appeared to be more relaxed than were the controls. In case of the salt-deficient rat 9 the large difference in heat production between the average of 4 hours of apparent inactivity and the average of all 8 hours is due to an abnormal condition of the animal. During the last 4 hours of observation the rat was not moving, but was breathing unusually fast and exhibited slight tremor, and the CO_2 production rose abnormally high. This rat died the following day.

The influence of the mineral deficiency on the basal metabolism

Accepting the general definition of basal metabolism as the metabolism measured under conditions of muscular repose

and when no food is being digested or absorbed, we have utilized for the determination of the basal metabolism of the rats the data representing the average hourly heat production of the fasting animals, excluding the hours of activity. For

TABLE 3

Influence of mineral-poor ration on basal metabolism

RAT NO.	BEFORE GIVING EXPERIMENTAL DIETS			DIET NO.	AFTER MAINTENANCE ON EXPERIMENTAL DIETS				DECREASE $\frac{(1)-(2)}{(1)}$
	Initial body weight	Calories[1]			Final body weight	Days on diet	Calories[1]		
		Per 100 gm. per hour	Per M^2 per 24 hours (1)				Per 100 gm. per hour	Per M^2 per 24 hours (2)	
	gm.	*cal.*	*cal.*		*gm.*		*cal.*	*cal.*	%
3	108	0.744	911	I	147	87	0.587	718	21.1
4	110	0.711	870	II	139	87	0.527	645	25.9
6	110	0.756	925	I	145	89	0.580	710	23.3
7	109	0.689	843	II	165	89	0.489	599	29.0
9	107	0.721	882	I	162	88	0.595	728	17.5
10	103	0.735	900	II	155	88	0.550	673	25.2
11	103	0.733	987	I	157	91	0.554	678	24.4
12	102	0.686	840	II	191	92	0.452	553	34.1
13	111	0.715	875	I					
14	104	0.724	886	II	190	87	0.428	524	40.9
15	103	0.767	939	I	111	84	0.631	772	17.7
16	110	0.738	903	II	157	84	0.510	624	30.9
19	111	0.724	886	I	153	85	0.557	682	23.1
20	102	0.717	878	II					
23	111	0.770	942	I	138	83	0.601	736	21.9
24	108	0.749	917	II	151	83	0.466	570	37.8
25	115	0.717	878	I	159	83	0.534	654	25.5
26	108	0.741	907	II	157	83	0.465	569	37.2
Average		0.739	914	I (low salt)			0.580	710	21.8
Average		0.721	882	II (control)			0.486	595	32.6

[1] Kilogram-calories.

the purpose of comparison we have expressed the results on the basis of 100 gm. body weight per hour, as well as per square meter of body surface per 24 hours. These data for the initial and final fasting periods are presented in table 3.

The computation of the metabolism to a basis of 100 gm. was made not in direct proportion of the body weight but in proportion to the two-thirds power of the body weight. In this manner recognition was given to the conception of an exponential relationship between energy metabolism and body weight.

The computation of the results per square meter of body surface was made on the basis of Meeh's formula, $S = 9.1\ W^{2/3}$, which was used by Benedict and MacLeod ('29 a, b) and adopted by Horst, Mendel and Benedict ('30, '34 a, b, c, d). The latter calculation was made not without the realization of the uncertainty of the determination of the surface area of the rat. The inaccuracies involved in the computation of the metabolism per square meter of body surface have been discussed by Benedict ('31–'32), Kleiber ('32) and Brody ('34).

Before the rats were placed on the experimental diets their basal metabolism was over 700 gram-calories per 100 gm. per hour and about 900 kilogram-calories per square meter per 24 hours, the results being fairly uniform. The mean of all individual determinations was found to be 730 calories per hour or 898 kilogram-calories per square meter per 24 hours, with an average deviation from the mean of $\pm 2.3\%$.

The respiratory quotients were also very uniform. The average of all determinations in the initial period was found to be 0.739 ± 0.005.

Contributing to this uniformity of the results are the facts that the animals were of the same strain, of the same sex, of approximately the same age and weight, and that they had been all kept on the same diet prior to this initial fasting period. The fact that the values were practically free from effects of activity is perhaps the most important factor in this relationship.

At the end of approximately 3 months (83 to 92 days) on the experimental diets a marked influence of the mineral deficiency on the basal metabolism is shown by the comparison of the results for the low-salt animals with those obtained with the controls. Without a single exception, the basal meta-

bolism of the salt-deficient animal was found to be considerably higher than the value for the control. The average basal metabolism of eight salt-deficient animals which survived, was found to be 710 calories per square meter per 24 hours as compared with 595 calories for the controls. The difference of 115 calories represents an increase in favor of the salt-deficient group of 19.3%. These results are highly significant and indicate that the elevated basal metabolic rate of the animals which received the low-salt diet was brought about by the mineral deficiency.

Very little or no effect was observed on the fasting respiratory quotients, since the final respiratory quotients with both groups of animals were identical, or nearly so, with those obtained in the initial fasting periods. This may be interpreted to signify that the effect of the mineral deficiency on the basal metabolism was only in respect to the intensity.

The influence of advancing age and suppression of growth

When the basal metabolism of the animals in the final fasting periods is compared with the metabolism in the initial periods (table 3, last column), it is observed that a marked reduction in the basal metabolic rate occurred with both groups of animals. This general decline in the basal metabolism, per unit of weight or size, represents the combined effect of advance in age and of the limited food intake. The effect of the mineral deficiency was to render this decline less pronounced. With the salt-deficient animals the decreases in basal metabolism ranged from 17.5% to 25.5%, averaging 21.8%, whereas with the controls these decreases ranged from 25.2% to 40.9% and averaged 32.6%.

The results obtained with rats 23, 24, 25 and 26, which are presented graphically in figure 1, are of especial interest in that they show the changes in the basal metabolism at approximately monthly intervals. These results show that the decline in metabolism was most pronounced during the first month and that it was significantly greater with the controls than with the low-salt animals. The basal metabolism of rat 23

(low-salt) declined during the first 31 days on the experimental diet from 942 calories per square meter per 24 hours to 783 calories—a decrease of 16.9%. During the same interval the decline in metabolism with rat 24 (control) was from 917 calories to 685 calories per square meter—a decrease of 25.3%. During the next 52 days the basal metabolism of rat 23 declined only 5.0% of the initial value, while the decline with rat 24 was 12.5%. Similarly, the basal metabolic rates

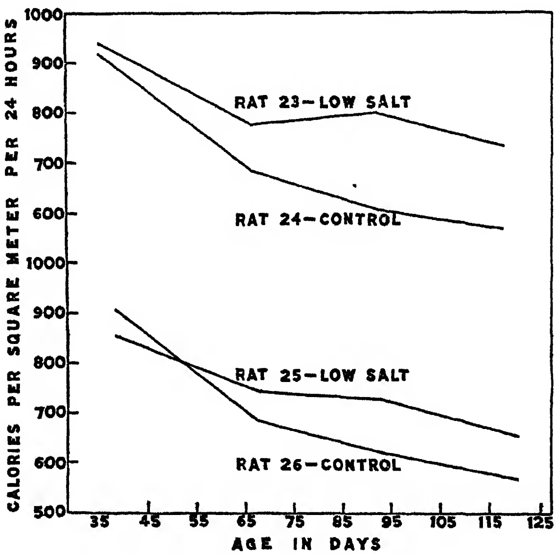

Fig. 1 The influence of advancing age and suppression of growth on the basal metabolism of rats receiving the low-salt diet, and of their controls.

of rat 25 (low-salt) and rat 26 (control) declined, respectively, 14.9% and 24.2% during the first 31 days, and 10.6% and 13.0% during the next 52 days.

It is clear from figure 1 that the response of the rats to the mineral deficient diet, insofar as it affects their basal metabolic rate, is relatively prompt. It is a significant fact that the greatest divergence between the growth curves of eight of the ten pairs of rats compared, occurred during the first month of the experimental dietary treatment, the low-salt animal

making the smaller gains in weight. The coincident rise, during this period, in the basal energy requirements of the animals receiving the low-salt diet, as compared with their controls, may serve to explain, to a large extent, the failure of the salt-deficient animals to utilize their diet for body increase as efficiently as did the controls.

DISCUSSION

It is a pertinent question to ask what the immediate cause for the increased basal metabolism of the salt-deficient rat is. We can give no direct answer to this question; as will be shown in a subsequent paper, the protein metabolism cannot account for it. The rectal temperature of the low-salt rats is consistently lower than that of their controls.[4] The increased metabolism of the former cannot, therefore, be directly attributed to body temperature. Brody ('32) has suggested that the basal metabolism of rats is proportional to the size of the visceral organs. Since some of the visceral organs, notably the kidney, of the low-salt rats were found to be enlarged (Swanson, Storvick and Smith, '36), this enlargement of the organs may be one of the causes for the increased basal metabolism.

In view, however, of the finding (Swanson and Smith, '32) that the low-salt diet brings about a condition of polycythemia with a reduction of the hemoglobin content of the blood—a constituent which plays a profound role in the processes of respiration—it seems more probable that the physico-chemical changes which have been found to take place in the blood as a result of the mineral deficiency are directly responsible for the marked alteration in the basal energy transformations of the animal. The very recent finding (Eppright, '36) that certain endocrine organs, notably the adrenals, are affected by the low-salt diet suggests the possibility that certain hormones are involved. This is a problem for future investigation.

[4] Unpublished observation of Dr. P. K. Smith.

SUMMARY AND CONCLUSIONS

The basal metabolism of young rats kept on a low-salt diet, as well as that of their calorie controls receiving an adequate supply of mineral salts, declined with increasing age during a period of approximately 3 months. The decline was most pronounced during the first month and was greater with the controls than with the low-salt animals. The rats kept on the mineral deficient diet were less active and invariably had a considerably higher basal metabolism than their controls. The salt deficiency apparently had no effect on the respiratory quotients of the fasting rats, which may be interpreted to indicate that the effect on the metabolism was only in relation to the intensity.

The authors express their appreciation to Dr. James Melville for valuable technical assistance in connection with this investigation.

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VITAMINS A, C AND D IN MAIZE AS AFFECTED BY VARIETY AND STAGE OF GROWTH [1, 2]

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This investigation was carried on to determine the effect of variety and stage of growth on the content of vitamins A, C and D in maize plants and kernels when grown under similar field conditions. Evidence is also presented to show that the comparative vitamin content of plant material is subject to a wide variation depending upon whether it is expressed on a moist or a dry basis.

The vitamin A content of maize kernels has been investigated by Fraps ('31), Hauge ('30), Mangelsdorf and Fraps ('31), Russell ('30), Steenbock and Boutwell ('20), Takahashi and Masuda ('35) and others; all of whom have found yellow maize superior to white maize in vitamin A content. The vitamin A content appears to vary directly with the number of genes for yellow pigmentation in the endosperm.

Ten varieties of maize: Canada Flint, Rhode Island Flint, Country Gentleman, Lancaster Sure Crop, Bantam Evergreen, Rustler's White, Golden Bantam, Black Mexican, Golden Sunshine and Stowell's Evergreen were grown in experimental plots on Merrimac fine sandy loam soil with 2000 pounds of 5-8-7- fertilizer mixture per acre. When the plants were approximately 25 cm. high a representative sample of the entire plant, except for the roots, was taken of the following varieties: Canada Flint, Rhode Island Flint, Country Gentleman,

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[2] Presented before the ninety-ninth meeting of the American Association for the Advancement of Science at Atlantic City, N. J., December 31, 1936.

Lancaster Sure Crop, Bantam Evergreen and Rustler's White. Later in the summer when the plants had attained their maximum growth and the kernels were at the milk stage, representative samples of the above six varieties were again taken and the percentage dry matter and vitamin A and C contents determined. The final samples were taken 1 month later, at which time the leaves and stalks had begun to turn brown. Fully or partially formed ears were not included in these plant samples. The vitamin A and moisture content of all the samples were determined. Vitamin C determinations were made on the first two series of samples. These data are presented in tables 1 and 2.

The percentage dry matter in each sample was determined by drying at 100 to 105°C. for 24 hours. The vitamin A content of each sample was determined by the Sherman and Munsell bio-assay method (Sherman and Smith, '31).

The rats previously depleted of vitamin A were used in series of eight at the several feeding levels for each variety. The samples of young corn plant were harvested each day and fed fresh over a 7-day period, then the samples were solidly frozen and used as needed for the rest of the feeding period. The other corn samples were frozen immediately after harvesting. Freezing and freezing storage are not known to injure vitamin A.

The immature kernels were fed fresh for 7 days and then sufficient was frozen for the remainder of the assay period. The mature kernels were dried and stored on the cob for 3½ months. The kernels were then ground to pass a 20-mesh sieve and kept in glass-stoppered bottles during the period of assay.

Vitamin C was determined by the 2, 6-dichlorophenolindophenol dye method of Tillmans as described by Bessey and King ('33), but modified by using for the extraction and acid solution 1 N with respect to sulfuric acid and N/4 with respect to metaphosphoric acid according to Mack, Tressler and Dearborn ('36). Comparisons made with the guinea pig assay method on immature sweet corn showed the chemical method to be reasonably accurate.

Vitamin D was determined by the usual U.S.P. ('34) assay method using Reference cod liver oil as a standard. Fresh plants and immature kernels were used in all but one series of the vitamin D assays. The exception consisted of corn plants which had been harvested and solidly frozen for 4 days before they were fed. This is explained in table 4.

EXPERIMENTAL RESULTS

From table 1 it is evident that there was a definite increase in the vitamin A and dry matter content of maize plants as they developed up to the full grown stage. There appears to be

TABLE 1

Effect of maturity of maize plants on vitamin A and moisture content

VARIETY	COLOR	DRY MATTER			VITAMIN A PER GRAM MOIST BASIS			VITAMIN A PER GRAM DRY BASIS			CONDITION OF MATURE PLANT
		Young	Full grown	Mature	Young	Full grown	Mature	Young	Full grown	Mature	
		%	%	%	I.U.	I.U.	I.U.	I.U.	I.U.	I.U.	
Canada Flint	Yellow	10.6	17.8	25.3	5.9	15.9	8.7	55.7	89.4	33.9	Green
Lancaster Sure Crop	Yellow	9.8	16.9	30.2	4.9	12.8	10.5	50.0	75.8	34.6	Green
Bantam Evergreen	Yellow	10.0	19.0	34.6	4.4	17.1	3.3	44.0	90.0	9.2	Dry
Rhode Island Flint	White	10.6	18.0	33.9	7.0	12.6	2.6	66.2	70.0	7.5	Dry
Country Gentleman	White	13.7	17.7	29.9	4.0	12.2	16.1	29.2	69.0	53.1	Green
Rustler's White	White	11.0	15.6	52.3	5.8	10.8	1.7	52.2	62.5	3.3	Dry
Average		10.9	17.5	34.3	5.3	13.5	7.1	49.5	76.0	23.6	

no definite correlation between the vitamin A content of the young maize plant and the amount of yellow color in the mature kernel. However, in the full grown plants, those bearing yellow kernels had a slightly higher vitamin A content. The higher vitamin A content of the full grown plants is actual and is not merely an apparent increase due to an increase in dry matter. The change in vitamin A content as the plant matures is much greater when calculated on a moist basis rather than on a dry basis. The mature plants showed a marked decrease in vitamin A content and an increase in dry

matter over the full grown plants. In the mature plants, however, the vitamin A content varied directly with the degree of greenness. The sample of Rustler's White which was very dry, showed a very marked loss of vitamin A. The variety highest in vitamin A content when young was Rhode Island Flint; when full grown, Bantam Evergreen was highest and Rustler's White was lowest.

The rapid loss of vitamin A in maize plants after they have attained maximum growth and tend to dry out is an important factor which should be taken into consideration when such material is used as a feed for livestock, either fresh or as silage. It is recommended that maize, when used for such purposes, should be harvested as soon as its full growth has been reached, in order to conserve its vitamin A content.

When the kernels of the Golden Bantam, Bantam Evergreen, Golden Sunshine, Country Gentleman, Stowell's Evergreen and Black Mexican were at the milk stage, (eating stage for the sweet corn varieties) and again after full maturity samples were taken and assayed for vitamins A and C. As shown in table 3, only the yellow varieties contained significant amounts of vitamin A. The three well-known sweet corn varieties, Golden Bantam, Bantam Evergreen and Golden Sunshine, at the immature eating stage, and at the mature stage contain significant amounts of vitamin A. On the other hand, the white and black varieties contained none or very little of this vitamin.

VITAMIN C

The vitamin C content of the young and full grown plants of the six varieties of maize, used in the vitamin A study, was determined and the data are presented in table 2. From these data it may be seen that the varietal difference in the vitamin C content of maize plants does not vary consistently with the stage of maturity of the plants. There is marked difference in the vitamin C content of the plants as they mature, depending upon whether it is calculated on a moist or a dry basis. On a dry basis all of the varieties exhibited a decrease

in the vitamin C content as the plants matured, but on a moist basis the vitamin C content increased in three of the varieties. There is no correlation between the amount of vitamin A and vitamin C present in maize plants, or between these vitamins and the degree of yellow pigmentation in the kernel.

Table 3 shows that sweet corn at the eating stage is a fairly good source of vitamin C. There is relatively little difference among the varieties examined. The average content of vitamin C is 3.1 I.U. per gram, e.g., 87.7 units per ounce. By comparison fresh orange juice contains approximately 250 units per ounce.

TABLE 2

Effect of maturity of maize plants on vitamin C and moisture content

VARIETY	COLOR	DRY MATTER		VITAMIN C PER GRAM MOIST BASIS		VITAMIN C PER GRAM DRY BASIS	
		Young	Full grown	Young	Full grown	Young	Full grown
		%	%	I.U.	I.U.	I.U.	I.U.
Canada Flint	Yellow	10.6	17.8	10.4	12.6	96.4	81.0
Lancaster Sure Crop	Yellow	9.8	16.9	9.5	18.0	96.8	81.0
Bantam Evergreen	Yellow	10.0	19.0	8.3	7.1	83.4	39.6
Rhode Island Flint	White	10.6	18.0	8.7	10.7	82.4	53.6
Country Gentleman	White	13.7	17.7	14.9	13.2	108.8	80.8
Rustler's White	White	11.0	15.6	....	16.4	....	95.0
Average		10.9	17.5	10.3	13.0	93.5	72.0

EFFECT OF REPORTING RESULTS ON A DRY BASIS

The data presented in tables 1 and 2 support the belief that the vitamin content of plant material should be calculated on a dry rather than on a moist basis. That the variable moisture content of plant material may be responsible for erroneous conclusions relative to the vitamin content is particularly evident from table 2. In this case, the results when calculated on a moist basis show that the vitamin C content of Canada Flint, Rhode Island Flint and Lancaster Sure Crop plants is substantially increased as the plants mature, but in reality there is a marked decrease in the vitamin C content of these varieties, as is shown by the data calculated on a dry basis. These same discrepancies due to variable

moisture content are evident, but not quite so marked, in the changes in the vitamin A content of maize plants, as may be seen in table 1.

As a result of these findings, the authors believe that in comparative vitamin studies, such as are here reported, the moisture content of the samples should be taken into consideration.

TABLE 3

Vitamin A, vitamin C and moisture content of maize kernels at the milk stage and mature stage

VARIETY	COLOR	DRY MATTER		VITAMIN A PER GRAM				VITAMIN C PER GRAM	
				Moist basis		Dry basis			
		Milk stage	Mature	Milk stage	Mature	Milk stage	Mature	Moist basis	Dry basis
		%	%	I.U.	I.U.	I.U.	I.U.	I.U.	I.U.
Golden Bantam	Yellow	36.2	91.1	6.2	6.8	17.3	7.4	3.0	8.4
Bantam Evergreen	Yellow	30.9	91.3	5.6	4.5	17.9	4.9	3.0	9.6
Golden Sunshine	Yellow	37.0	91.0	4.2	2.4	11.3	2.6	3.2	8.6
Country Gentleman	White	18.5	90.9	[1]	[1]	[1]	[1]	[2]	[2]
Stowell's Evergreen	White	21.6	91.9	[1]	[1]	[1]	[1]	[2]	[2]
Black Mexican	Purple	29.5	90.8	[1]	[1]	[1]	[1]	[2]	[2]
Average (for three yellow var.)		34.7	91.1	5.3	4.5	15.5	4.9	3.1	8.8

[1] Less than 1.0 unit per gram. When fed 1.5 gm. daily failed to prevent weight losses.

[2] Vitamin C not determined in these varieties.

VITAMIN D

Harris and Bunker ('34) have found samples of fresh yellow maize from different localities to show marked variations in their rachitogenic properties, which are due apparently to the presence of an antirachitic substance. Templin and Steenbock ('33) noted that immature yellow dent field maize kernels promoted better calcification than corresponding mature maize. Bechtel and Hoppert ('36) investigated the vitamin D contents of dry tassels, silk and leaves of the maize plant at the time of ensiling and found that on a basis of D units per pound of dry matter the tassels, silk and dry leaves contained 1226, 2449 and 2449 units of vitamin D, respectively. Coward

('32) has found that the antirachitic property of green plant material is rapidly lost on storage.

The samples of mature plants which had been frozen for 4 days contained only a very small amount or a trace of vitamin

TABLE 4

Vtiamin D content of maize

VARIETY	COLOR	AMOUNT FED DAILY	NUMBER OF RATS	AVERAGE DEGREE OF CALCIFICATION	VITAMIN D PER GRAM MOIST BASIS
Samples frozen 4 days					
		gm.			*I.U.*
Canada Flint	Yellow	1.0	5	0.6	Trace
Lancaster Sure Crop	Yellow	1.0	5	2.0	2.7–
Bantam Evergreen	Yellow	1.0	5	1.3	Trace
Rhode Island Flint	White	1.0	5	0.1–	Trace–
Country Gentleman	White	1.0	5	0.0	None
Rustler's White	White	1.0	5	0.4	Trace
Samples taken fresh daily					
Canada Flint	Yellow	1.0	8	3.0	2.7
Lancaster Sure Crop	Yellow	1.0	8	2.6	2.7–
Bantam Evergreen	Yellow	1.0	8	2.7	2.7–
Rhode Island Flint	White	1.0	8	2.7	2.7–
Country Gentleman	White	1.0	8	3.0	2.7
Black Mexican	Purple	1.0	6	3.0	2.7
Immature kernels					
Gonden Bantam	Yellow	1.0	6	0.5	Trace
Bantam Evergreen	Yellow	1.0	8	0.0	None
Gonden Sunshine	Yellow	1.0	8	0.1	Trace–
Country Gentleman	White	1.0	8	0.2	Trace
Stowell's Evergreen	White	1.0	8	0.0+	None+
Black Mexican	Purple	1.0	6	0.3	Trace
Positive control	28.5 mg. U.S.P. reference cod liver oil		10	2.9	95
Negative control			5	0.0	None

D whereas the fresh plants contained at least 2.7 I.U. of vitamin D per gram moist or 15 units on a dry basis. This value is equivalent to 6700 units per pound of dry matter. The fresh kernels contained only a trace of vitamin D. These results are in agreement with unpublished data of Coward ('32) who found that the vitamin D content of green plant material was lost rapidly on storage.

CONCLUSIONS

1. There is a definite increase in the vitamin A content of maize plants as they grow. Average values for 25 cm. plants and plants after they had attained maximum growth were 49 and 76 units per gram, respectively (dry basis).

2. The vitamin C content of these same maize plants decreased from 93.5 to 72 units per gram (dry basis).

3. There is no correlation between the vitamin A and C content of the plant and the color of the kernels of maize plants.

4. Only the yellow pigmented kernels of maize plants contain demonstrable amounts of vitamin A.

5. Fresh maize plants contain an antirachitic substance which is lost rapidly on storage. The fresh immature or ripe grain kernels contain only a trace of vitamin D.

6. The importance of reporting vitamin data in plant material in terms of dry matter, in order to avoid possible erroneous conclusions, is stressed.

7. There is a marked loss in vitamin A and moisture in maize plants after they have attained maximum growth. This loss of vitamin A is a factor which should be taken into consideration when maize is grown as a feed for livestock.

8. Kernels of yellow maize lose much of their vitamin A as they mature and dry out.

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THE EFFECT OF THE ORAL ADMINISTRATION OF PANCREATIN ON FECAL NITROGEN AND FAT LOSS IN ACHYLIA PANCREATICA

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In a previous publication we have shown that the oral administration of diastase decreases the starch lost in the feces in achylia pancreatica (Beazell, Schmidt and Ivy, '37). A review of the literature reveals that complete exclusion of pancreatic juice from the intestine increases the loss of fat and nitrogen in the feces (Pratt, Lamson and Marks, '09; Cruickshank, '15; Maltby, '31; Nasset, Pierce and Murlin, '31; Handelsman, Golden and Pratt, '34; Ivy, '35; and Selle, '37) and that substitution therapy has been uniformly effective in reducing the loss of nitrogen (Pratt, Lamson and Marks, '09; Cruickshank, '15; Nasset, Pierce and Murlin, '31; and Selle, '37). The value of orally administered enzymes in reducing the loss of fat remains, however, unsettled (Selle, '37). In this study we have investigated the effect of oral enzyme therapy on the loss of nitrogen and fat in the feces in pancreatic achylia.

METHODS

In seven dogs weighing from 11 to 14 kilos the pancreatic juice was totally excluded from the intestine by separating the pancreas from all of its connections with the duodenum. Post-operatively the dogs were fed a nutritious diet containing 200 gm. of raw pancreas for a period of a month or longer. This diet maintained them in excellent condition.

Submaintenance diet. Five of the animals were then given a submaintenance diet consisting of 200 gm. of raw, lean meat daily for a period of 21 days. The feces were discarded for the first 2 days after which collections were made for the next 5 days. This constituted the first control period. Twenty-five grams of pancreatin, in the form of enteric coated tablets, were then added to the daily diet of each dog. Forty tablets were given with the meal, twenty more at 2 hours, ten at 4 hours, and five at 6 hours post-cibum. The feces were again discarded for the first 2 days and collected for the following 5 days. This constituted the 'pancreatin' period. The pancreatin was then discontinued, and after discarding the feces for 2 days, collections were again made for 5 days. This constituted the second control period. The total and dry weights and the nitrogen content of the feces was determined daily. Nitrogen determinations were made in duplicate on the dried feces by the Kjeldahl method.

Maintenance diet. The animals were then returned to the diet containing raw pancreas for several months. Dogs nos. 3 and 4 used above and two dogs not previously used were then given a standard diet of 600 gm. of cooked ground beef heart from which all visible fat had been removed. The diet was prepared in 50 pound batches and was preserved in the ice box. Nitrogen determinations were made in duplicate on aliquots from each batch.

As before the experiment was divided into three 5-day metabolic periods. During the first and last periods the feces were collected while the animals were on the diet (control periods). During the second period 25 gm. of pancreatin, in the form of enteric coated tablets, were given in a single dose with the daily diet. A carmine 'marker' was given mixed with the meal on the third day of each period and again on the eighth day. The feces were collected daily starting with the first appearance in the stool of the first dose of carmine and ending with the first appearance of the second dose.

The 24-hour excretion of feces from each dog was weighed daily and, after thorough mixing, aliquots were taken for the

fat and nitrogen determinations. The samples for each dog were pooled during each metabolic period and determinations were made on the composite sample. The samples for the nitrogen determination were suspended in sulphuric acid as recommended by Peters and Van Slyke ('32); samples for the fat determination were preserved in alcohol. Nitrogen was determined by the Kjeldahl method, fat by a modification of Saxon's method (Peters and Van Slyke, '32). All determinations were made in duplicate. No correction was made for the bulk or metabolite content of the pancreatin.

The tryptic activity of the pancreatin used throughout this study was from 50% to 100% greater than that required by the U.S.P. The lipase activity was determined by the method described by Cherry and Crandall ('32). The activity, expressed as the number of cubic centimeters of 0.2 N. NaOH required to neutralize the fatty acids liberated by 1 gm. of pancreatin acting at optimum concentration, was 2200 units.

RESULTS

Control periods. The feces passed during the control periods were, in most instances, bulky and characteristic of pancreatic achylia. The individual nitrogen excretion values checked remarkably well for the first and third control metabolic periods. On the maintenance diet the average nitrogen utilization was 69.2% for the first period, and 71.5% for the third period (table 2). Fecal fat values showed greater individual variations, although averages for the two periods compare favorably. During the first 5-day control period an average of 50.0 gm. of fat (14.6 to 78.7 gm.) was eliminated; during the third period, 47.0 gm. (9.9 to 86.1 gm.) (table 3).

Pancreatin period. The addition to the diet of 25 gm. of enteric coated pancreatin tablets had an inconstant effect on the dry weight of the feces. In the experiment in which dry weight was determined, three of the dogs showed an increase while the other two showed a decrease (table 1). The effect of pancreatin on the wet weight of the feces was more constant. On the submaintenance diet the wet weight of the feces was

TABLE 1

Effect of pancreatin on fecal nitrogen excretion. Five dogs with pancreatic achylia on a submaintenance diet (values are totals for entire metabolic period)

DOG NO.	First metabolic period 5 days—control				Second metabolic period 5 days—25 gm. pancreatin daily							Third metabolic period 5 days—control			
	Wet weight feces	Dry weight feces	Per cent solids	Total fecal nitrogen	Wet weight feces	Dry weight feces	Per cent solids	Total fecal nitrogen	Per cent change solids[1]	Per cent change bulk[1, 2]	Per cent decrease nitrogen[1]	Wet weight feces	Dry weight feces	Per cent solids	Total fecal nitrogen
1	1013	261	25.7	18.1	691	333	48.2	8.6	+18.0	—41.0	60.0	1330	304	22.9	25.2
2	1463	438	29.9	32.3	474	199	42.1	12.0	—53.8	—63.3	62.5	1120	425	37.9	31.7
3	480	137	28.5	13.4	545	234	42.9	6.1	+61.5	+4.2	57.2	567	153	27.0	14.8
4	135	49.5	36.6	5.0	341	131	38.4	4.1	+150.0	+154.0	21.0	133	55	41.3	5.5
5	1006	254	25.2	22.3	502	197	39.3	6.7	—12.4	—46.7	65.8	874	197	22.6	17.2
Average	819	228	27.9	18.2	510	219	42.9	7.5	—3.74	—37.2	59.5	805	227	28.2	18.8

[1] Based on average of two control periods.

[2] Based on wet weight of feces.

TABLE 2

Effect of pancreatin on fecal nitrogen and bulk. Four dogs with pancreatic achylia on a maintenance diet (values are totals for entire metabolic period)

DOG NO.	First metabolic period 5 days—control				Second metabolic period 5 days—25 gm. pancreatin daily						Third metabolic period 5 days—control			
	Wet weight feces	Nitrogen ingested	Fecal nitrogen	Nitrogen utilized	Wet weight feces	Nitrogen ingested	Fecal nitrogen	Nitrogen utilized	Change in bulk[1]	Reduction in N loss[1]	Wet weight feces	Nitrogen ingested	Fecal nitrogen	Nitrogen utilized
		gm.	*gm.*	%		*gm.*	*gm.*	%	%	%		*gm.*	*gm.*	%
3	597	110	22.1	79.5	421	107.7	13.2	87.7	—26.2	35.3	545	113	18.7	83.5
4	280	110	13.8	87.4	340	107.7	8.7	91.7	+12.9	31.5	322	113	11.6	89.7
6	1184	110	49.3	55.4	479	107.7	13.4	87.6	—59.5	73.4	1181	113	49.9	56.0
7	1142	110	50.6	54.1	584	107.7	18.9	82.5	—46.5	61.8	1046	113	48.4	57.3
Average	801	110	33.9	69.2	456	107.7	13.5	87.5	—42.2	59.2	773	113	32.2	71.5

[1] Based on average of two control periods.

decreased in only three of the five dogs studied, but the average change indicates a substantial decrease (37.2%). On the maintenance diet three dogs showed a decrease and one an increase, the average being a 42.2% decrease. Fecal nitrogen and fat were decreased significantly in all of the animals studied. Nitrogen loss was reduced 21 to 66% (average 59.5%) on the submaintenance diet and 31 to 73% (average 59.2%) on the maintenance diet (tables 1 and 2). Fecal fat loss was reduced 14 to 78% (average 59.1%) (table 3).

It will be seen by comparing the values in tables 1 and 2 that orally administered pancreatin exerts its greatest effect in those animals showing the greatest nitrogen and fat loss

TABLE 3

Effect of pancreatin on fecal fat excretion. Maintenance diet (600 gm. lean beef heart daily)

DOG NO.	TOTAL LIPOID IN FECES (GM.)			PERCENTAGE REDUCTION IN FAT LOSS EFFECTED BY PANCREATIN (TWO CONTROL PERIODS AVERAGED)
	First metabolic period (control)	Second metabolic period (pancreatin)	Third metabolic period (control)	
3	33.4	15.6	19.4	40.8
4	14.6	10.4	9.9	14.7
6	78.7	17.9	86.1	78.3
7	73.0	35.3	72.6	51.5
Average	50.0	19.5	47.0	59.1

during the control periods (dogs 1, 2, 5, 6, 7), and the least effect in those animals which tolerate pancreatic achylia well (dogs 3, 4). Dogs 3 and 4 have been maintained for a year and a half with pancreatic achylia and may represent an adaptation that occurs under such conditions. This seems likely in the case of dog 3 which on a submaintenance diet showed a fairly high nitrogen excretion that was decreased 57.2% by pancreatin (table 1). Nine months later, on a maintenance diet this animal showed a relatively low nitrogen excretion which was reduced only 35.3% when pancreatin was added to the diet. In any event, the nitrogen and fat utilization in all of the animals reached approximately the same level when adequate substitution therapy was instituted.

DISCUSSION

In depancreatized dogs, according to Cruickshank ('15), the administration of raw pancreas (150 gm.) increased nitrogen utilization from a control value of 78% to 92%. Our findings on the effect of pancreatin on nitrogen loss in dogs deprived of the external pancreatic secretion confirm previous reports. Nasset, Pierce and Murlin ('31) reported that pancreatin increased nitrogen utilization in two depancreatized dogs from a control value of 62% to 72% in one, and from 78% to 85% in the other. Selle ('37) reported that pancreatin decreased nitrogen loss from 30 to 60% in ten diabetic dogs maintained on insulin. In our studies of four dogs on a maintenance diet the average nitrogen utilization was 70.5% without pancreatin and 87.5% with pancreatin. The average reduction in nitrogen loss was 59.5% in five dogs on a submaintenance diet and 59.2% in four dogs on a maintenance diet.

Our findings on the effect of pancreatin on fat excretion in pancreatic achylia are in contrast to those of Selle who reported that pancreatin had no effect on fat excretion in diabetic dogs. In our dogs, the average reduction in fecal fat loss was 59.1%, variations from animal to animal being quite marked. Since an appreciable amount of fecal fat represents endogenous fat which is essentially unrelated to the fat of the diet, and since fat mobilization in diabetic animals, even when controlled with insulin, is abnormal, it is not unlikely that pancreatin would effect fecal fat differently in diabetic and nondiabetic dogs. The fed fat would have to be 'labeled' with heavy hydrogen to answer this question.

On the basis of these findings, together with those on starch digestion reported by us in a previous paper (Beazell, Schmidt and Ivy, '37), it would appear that pancreatin decreases to a comparable degree the loss of protein, fats, and starches in pancreatic achylia (50 to 60%). The decrease in moisture content of the feces by pancreatin may be due to either an osmotic effect or a chemical irritative effect exerted by the undigested or partially digested foods.

SUMMARY AND CONCLUSIONS

The pancreatic juice was completely excluded from the intestine in seven dogs. For a period of a month or more the animals were maintained in excellent condition on a mixed diet and raw pancreas. Five dogs were then placed on a submaintenance diet of 200 gm. lean beef for three consecutive 7-day periods (21 days). Pancreatin (25 gm.; potency two times U.S.P.) was given during the second 7-day period. This procedure was repeated with four dogs on a maintenance diet of 600 gm. of trimmed beef heart. Analysis of the feces for nitrogen and fat showed that pancreatin decreased the elimination of these constituents about 60% and 59%, respectively, regardless of the quantity of diet fed. Fecal bulk was reduced 37 to 42%, and the stools became better formed and drier. It is concluded that potent pancreatin, when given in the form of enteric coated tablets and in adequate amount is significantly effective in reducing nitrogen and fat loss in the feces of dogs having achylia pancreatica.

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THE UTILIZATION OF ENERGY PRODUCING NUTRIMENT AND PROTEIN AS AFFECTED BY DEFICIENCY OF IRON AND COPPER [1]

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The deficiency of milk in iron and copper having been first demonstrated by Hart, Steenbock, Waddell and Elvehjem ('28) to be the cause of the whole milk anemia of the albino rat, the study to be discussed was an investigation of these associated nutritive deficiencies, and the resulting anemia, as they affect the efficiency of utilization of food energy and protein.

In this relation the concern of the authors with the development of symptoms of anemia was only as this condition bore definite evidence that the iron and copper deficiency in the food, the effects of which were to be studied, had become nutritionally effective.

In the production of milk anemia in young rats Elvehjem and Kemmerer ('31) have recommended that they be prevented from consuming any food other than milk, during the suckling period; and Levine, Culp and Anderson ('32) placed the rats on a milk diet as soon as the average body weight of the animal reached 40 gm. (approximately 21 days of age), in order to diminish body stores of iron and copper as rapidly as possible.

A spray process, whole milk powder (Klim) has been found more convenient than liquid whole milk, for this purpose, by

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Harris ('32), Levine, Culp and Anderson ('32), and Remington ('33), since a sufficient quantity of the product, of uniform composition, can be obtained to last for an entire experiment. These workers report satisfactory results from feeding the milk powder in a dry condition; and this method of feeding milk is especially desirable when an accurate record of the food consumption is wanted. The group of workers above mentioned report the iron content of the milk powder as varying between 2.4 and 5 parts per million, and the copper content between less than 1 and 2 parts per million.

Harris ('32) observed that rats fed a ration of whole milk powder (Klim) developed diarrhea, which usually cleared up by the seventh day after weaning. He attributed this condition to the high lactose content of milk.

Palmear and Kennedy ('31) found the digestibility of whole milk powder to be slightly higher than that of liquid milk, the values being 95.6% and 92.7%, respectively.

The prolonged feeding of a milk-iron-copper diet to rats was found by Underhill, Orten, Mugrage and Lewis ('33) to be without harmful effects, though the body weight attained was somewhat less than normal.

Among the observations which have been made on the growth of rats with nutritional anemia, Elvehjem and Kemmerer ('31) were able to produce severe anemia within 2 weeks after weaning, when special precautions were observed not to allow the young rats to receive any food other than liquid milk. The body weight of these animals increased very little after they were weaned; and death usually resulted by the time they had been weaned 4 weeks.

Supplee, Dow, Flanigan and Kahlenberg ('30) found that rats which received reconstituted, spray process, dry milk, and had initial weights of approximately 45 gm., and blood hemoglobin of 14 gm., increased in weight to 110 gm. during 10 weeks, the hemoglobin value decreasing to about 4.5 gm. during this time.

Harris ('32) fed a group of 100 weanling albino rats for a period of 9 weeks on a whole milk powder (Klim) ration. The

approximate initial and final live weights of the rats (as indicated in a graph) were 35 and 170 gm., and the blood hemoglobin values 11.0 and 2.8 gm., respectively.

Remington ('33) states that the inverse relationship between rate of growth and blood hemoglobin value, as reported by Harris, is in agreement with the findings of others. Andes and Beard ('34) found that ten rats, which ingested an average of 38 cc. of milk per day, increased in weight from 92.0 gm. to 160.5 gm. in 10 weeks. During the same period the hemoglobin content of the blood decreased from 3.9 gm. to 3.6 gm. per 100 cc.

It has been reported by Krauss ('31) that rats fed exclusively on whole milk had enlarged hearts, lungs, spleens and kidneys, but smaller testes than normal. The observation of hypertrophy of cardiac muscle has been confirmed by Daniels and Burright ('33), who found this condition continuing after the rats had been cured of the anemia.

The experiment to be reported was devised as a unit in a series of investigations of the efficiency of food utilization as affected by specific nutrient deficiencies—in this case, of iron and copper; this phase of nutritional anemia not having been investigated, heretofore, by carefully controlled experimentation.

The method of procedure followed was the same as in previous studies of this series, and as described by Swift, Kahlenberg, Voris and Forbes ('34).

PRELIMINARY EXPERIMENT

In the first start of this experiment a diet of whole milk powder (spray process), dextrin, casein, Crisco and vitamin B concentrate was fed. It was found, however, that the dextrin and Crisco contained considerable amounts of iron; and on this account the rats did not develop nutritional anemia. This false start, therefore, was abandoned, and the experiment was begun again on the better basis to be described.

EXPERIMENTAL PROCEDURE

Two rations of whole milk powder (Klim), one unsupplemented, and the other supplemented with iron and copper, were compared in a growth, metabolism and body balance study with twelve pairs of weanling albino rats. The pair mates were of the same sex, from the same litter, and of approximately the same body weight at the start of the experiment.

In order to develop the nutritional anemia as rapidly as possible the mothers of the experimental subjects were fed milk powder alone from the time the young rats were 7 days of age until they were weaned at the age of 21 days. Also, the experimental cages and feeders were lacquered, and the animals were not allowed access to any copper or iron; and, further, all water given the rats while on experiment was redistilled in glass.

TABLE 1

Gain in body weight[1] of rats as related to dry matter of food

PAIR	SEX	Fe + Cu DEFICIENT DIET			Fe + Cu SUPPLEMENTED DIET		
		Food eaten (dry matter)	Gain in body weight	Dry matter of food per gram body gain	Food eaten (dry matter)	Gain in body weight	Dry matter of food per gram body gain
		gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
1	♂	187.4	37.65	5.0	187.1	45.21	4.1
2	♂	192.4	46.99	4.1	192.1	56.70	3.4
3	♀	202.4	50.61	4.0	202.1	52.95	3.8
4	♂	187.0	43.37	4.3	186.7	56.93	3.3
5	♂	174.2	42.99	4.1	173.9	53.52	3.2
6	♂	184.1	52.88	3.5	183.8	58.98	3.1
7	♀	182.8	35.43	5.2	182.5	42.62	4.3
8	♀	200.3	41.62	4.8	200.0	43.11	4.6
9	♂	183.0	36.85	5.0	182.7	44.49	4.1
10	♀	183.5	43.46	4.2	183.2	45.15	4.1
11	♀	202.5	51.53	3.9	202.2	50.82	4.0
12	♀	200.5	46.85	4.3	200.2	52.44	3.8
Average		190.0	44.19	4.3	189.7	50.24	3.8

[1] Excluding contents of alimentary tract.

Since most of the rats had diarrhea when weaned, they were not started on experiment until 26 days of age (average)

in order to allow time for this disorder to clear up. Some trouble on this account continued, with a few of the rats, however, the diarrhea persisting, in a mild form, for from 4 to 5 weeks.

TABLE 2

Energy of body gain of rats as related to energy, and to metabolizable energy of food

PAIR	FEED ENERGY	Fe + Cu DEFICIENT DIET				Fe + Cu SUPPLEMENTED DIET			
		Body gain		Metabolizable energy[1]	Body gain as percentage of metabolizable energy	Body gain		Metabolizable energy[1]	Body gain as percentage of metabolizable energy
			Per cent of feed energy				Per cent of feed energy		
	Cal.	*Cal.*		*Cal.*	%	*Cal.*		*Cal.*	%
1	1054.2	56.8	5.4	930.9	6.1	75.0	7.1	917.4	8.2
2	1082.5	87.8	8.1	953.0	9.2	139.4	12.9	961.8	14.5
3	1138.9	111.7	9.8	1020.3	10.9	140.2	12.3	1011.9	13.9
4	1052.0	72.6	6.9	928.3	7.8	137.5	13.1	921.4	14.9
5	980.1	87.9	9.0	877.1	10.0	131.8	13.4	871.4	15.1
6	1036.0	106.2	10.3	938.2	11.3	146.9	14.2	915.1	16.1
7	1028.2	87.2	8.5	914.8	9.5	101.7	9.9	881.0	11.5
8	1126.7	78.7	7.0	983.5	8.0	116.3	10.3	954.1	12.2
9	1029.3	50.8	4.9	945.4	5.4	97.3	9.5	899.4	10.8
10	1032.1	112.2	10.9	930.6	12.1	103.9	10.1	902.4	11.5
11	1139.5	94.8	8.3	1040.6	9.1	153.2	13.4	977.2	15.7
12	1127.8	102.7	9.1	984.3	10.4	168.2	14.9	1007.9	16.7
Ave.	1068.9	87.5	8.2	953.9	9.2	126.0	11.8	935.1	13.5

[1] Uncorrected for non-metabolizable energy of protein gained.

The same lot of whole milk powder (Klim) was used in both rations during the entire experiment. The milk powder was of a special lot which had been recommended by Remington (personal communication) as being consistently low in iron and copper.

The supplemented ration contained in each 5 gm. 0.5 mg. of iron in the form of $Fe\ Cl_3 .6H_2O$, and 0.05 mg. of copper in the form of $CuSO_4.5H_2O$.

The average food consumption was somewhat less than 5 gm. per day, the smallest average being 4.2 gm. However, this amount of feed provided enough of the iron and copper supplements to cure nutritional anemia.

The food intake was controlled by the paired method, and the two rations contained equal amounts of nitrogen and energy per gram.

The experiment was brought to a close, on account of marked decrease in food consumption, after 38 days' feeding of pair no. 9, and 42 days' feeding of the remaining eleven pairs.

At the end of the experiment the rats were killed, and chemical analyses were made of the rat bodies, and of the food, urine and feces.

TABLE 3

Heat loss related to energy of food

PAIR	FEED ENERGY	Fe + Cu DEFICIENT DIET		Fe + Cu SUPPLEMENTED DIET	
		Heat loss		Heat loss	
			Per cent of feed energy		Per cent of feed energy
	Cal.	*Cal.*		*Cal.*	
1	1054.2	874.1	82.9	842.4	79.9
2	1082.5	865.2	79.9	822.4	76.0
3	1138.9	908.6	79.8	871.7	76.5
4	1052.0	855.7	81.3	783.9	74.5
5	980.1	789.2	80.5	739.6	75.5
6	1036.0	832.0	80.3	768.2	74.2
7	1028.2	827.6	80.5	779.3	75.8
8	1126.7	904.8	80.3	837.8	74.4
9	1029.3	894.6	86.9	802.1	77.9
10	1032.1	818.4	79.3	798.5	77.4
11	1139.5	945.8	83.0	824.0	72.3
12	1127.8	881.6	78.2	839.7	74.5
Average	1068.9	866.5	81.1	809.1	75.7

The body gains of nitrogen and energy and the heat production were computed by a difference procedure. To obtain the composition of the rat bodies at the start of the experiment, a group of control animals, of similar weight and treatment, was killed and analyzed at this time. The gains of nitrogen and energy were computed as the difference between the final analyses of the experimental animals and the estimated amount of nitrogen and energy which these animals contained at the start of the experiment. The heat production was computed by subtracting from the gross energy of the feed the sum of the energy equivalents of the feces, urine and body gain.

The experimental procedure was as described, in detail, by Swift, Kahlenberg, Voris and Forbes ('34), and by Forbes, Swift, Black and Kahlenberg ('35).

TABLE 4

Nitrogen of body gain related to fat and energy of body gain, and to nitrogen of feed

PAIR NO.	NITROGEN OF BODY GAIN	Fat gained		Energy gained			Nitrogen of feed	
		Total	Per gram nitrogen gained	Total	As protein	As fat		Utilized for body gain
				Fe + Cu DEFICIENT DIET				
	gm.	*gm.*	*gm.*	*Cal.*	*Cal.*	*Cal.*	*gm.*	*%*
1	1.54	0.6	0.4	56.8	51.3	5.5	7.37	20.9
2	1.64	3.6	2.2	87.8	53.8	34.0	7.57	21.7
3	1.77	5.7	3.2	111.7	58.3	53.4	7.96	22.2
4	1.45	2.7	1.9	72.6	47.7	24.9	7.36	19.7
5	1.43	4.3	3.0	87.9	47.2	40.7	6.85	20.9
6	1.73	5.2	3.0	106.2	57.4	48.8	7.24	23.9
7	1.09	5.6	5.1	87.2	35.4	51.8	7.19	15.2
8	1.49	3.4	2.3	78.7	46.8	31.9	7.88	18.9
9	1.38	0.6	0.4	50.8	44.9	5.9	7.20	19.2
10	1.48	6.6	4.5	112.2	50.5	61.7	7.22	20.5
11	1.78	3.9	2.2	94.8	59.2	35.6	7.97	22.3
12	1.41	5.8	4.1	102.7	48.4	54.3	7.89	17.9
Average	1.52	4.0	2.6	87.5	50.1	37.4	7.48	20.3
				Fe + Cu SUPPLEMENTED DIET				
1	1.89	1.3	0.7	75.0	62.4	12.6	7.37	25.6
2	2.03	7.7	3.8	139.4	67.0	72.4	7.57	26.8
3	1.77	8.5	4.8	140.2	60.9	79.3	7.96	22.2
4	1.99	7.4	3.7	137.5	68.8	68.7	7.36	27.0
5	1.92	7.2	3.8	131.8	63.6	68.2	6.85	28.0
6	2.04	8.0	3.9	146.9	71.5	75.4	7.24	28.2
7	1.54	5.2	3.4	101.7	53.0	48.7	7.19	21.4
8	1.50	6.6	4.4	116.3	54.2	62.1	7.88	19.0
9	1.80	4.0	2.2	97.3	59.3	38.0	7.20	25.0
10	1.70	5.2	3.1	103.9	55.0	48.9	7.22	23.5
11	1.74	10.2	5.9	153.2	58.5	94.7	7.97	21.8
12	1.72	11.8	6.9	168.2	58.2	110.0	7.89	21.8
Average	1.80	6.9	3.8	126.0	61.0	64.9	7.48	24.1

Iron was determined in the milk powder by the method of Elvehjem, as described by Farrar ('35), and copper by the potassium ethyl xanthate method which was adopted for determination of copper in milk by Supplee and Bellis ('22).

The hemoglobin content of the blood was determined at the end of the second, third and sixth weeks of the experiment, by the colorimetric acid hematin method of Newcomer, with a glass standard.

TABLE 5

Digestibility of nitrogen and energy producing nutriment

PAIR	Fe + Cu DEFICIENT DIET				Fe + Cu SUPPLEMENTED DIET			
	Nitrogen		Energy		Nitrogen		Energy	
	Feces	Digested	Feces	Digested	Feces	Digested	Feces	Digested
	gm.	*%*	*Cal.*	*%*	*gm.*	*%*	*Cal.*	*%*
1	0.63	91.5	75.3	92.9	0.92	87.5	97.0	90.8
2	0.66	91.3	84.7	92.2	0.75	90.1	81.0	92.5
3	0.54	93.2	72.2	93.7	0.75	90.6	82.1	92.8
4	0.67	90.9	75.1	92.9	0.82	88.9	85.9	91.8
5	0.67	90.2	63.5	93.5	0.72	89.5	73.2	92.5
6	0.60	91.7	56.0	94.6	0.80	89.0	84.5	91.8
7	0.60	91.7	57.0	94.5	0.89	87.6	93.0	91.0
8	0.72	90.9	84.6	92.5	1.00	87.3	118.4	89.5
9	0.46	93.6	37.7	96.3	0.86	88.1	92.1	91.1
10	0.71	90.2	52.2	94.9	0.97	86.6	87.7	91.5
11	0.64	92.0	50.5	95.6	1.10	86.2	114.4	90.0
12	0.60	92.4	61.7	94.5	0.83	89.5	73.8	93.5
Average	0.63	91.6	64.2	94.0	0.87	88.4	90.3	91.6

The energy content of both diets was determined, by analysis, to be 5534 Calories per gram, and the nitrogen content, 3.87%. The moisture content of the deficient ration was 1.63%, and of the supplemented ration, 1.79%. The iron and copper contents of the whole milk powder were 3 parts, and 1½ parts per million, respectively.

PRESENTATION OF RESULTS

There was a total of 242 refusals of feed during the course of the experiment, as food was given each pair of rats until one individual refused. Of these refusals 225 were by the rats which received the deficient diet, and only seventeen by the rats on the supplemented diet, a difference that would occur by chance once only in billions of trials. Thus the anemic rats very definitely restricted the food intake of their pair mates.

The average gains in body weight, excluding the contents of the alimentary tract, were 44.19 gm. for the deficient rats, and 50.24 gm. for the supplemented rats. Statistical treatment reveals odds of 5000 to 1 that the gain made by the supplemented rats was significantly greater than that made by the deficient rats. Since the energy intake of each pair of rats was identical, there is a correspondingly more efficient utilization of food by the supplemented rats, which is represented in the dry matter of food per gram of body gain.

The energy storage by the supplemented rats was greater than by the deficient rats in eleven pairs among the twelve. The average energy gains were 126.0 Calories for the supplemented rats, and 87.5 Calories for the deficient rats; and the odds that this difference is significant are over 10,000 to 1. The difference between the metabolizable energy values of the diets for the two groups of rats was very small, but the odds that this difference was significant were 100 to 1. Consequently there was a higher percentage of metabolizable energy utilized for body gain by the supplemented than by the deficient rats.

The values reported for metabolizable energy in this experiment were not corrected for the non-metabolizable fraction of the energy stored as protein. The derivation of these values is simply the gross energy of the feed minus the energy of the feces and urine.

The iron-and-copper deficient rats, in a state of nutritional anemia, produced more heat in every case than did their pair mates, the blood of which was maintained normal by the addition of iron and copper to the diet. The significance of this difference is expressed by odds of over 10,000 to 1.

The group of rats which gained the most energy (the supplemented rats) must necessarily have produced less heat than the other, since both groups of animals metabolized essentially the same quantities of food energy.

It is possible that the increased heat production of the deficient animals resulted either from increased activity, or from increased energy expense of supplying the tissues with oxygen.

Of the energy retained by the deficient rats, 50.1 Calories were gained as protein, and 37.4 Calories as fat; while with the supplemented rats, 61.0 Calories were gained as protein, and 64.9 Calories as fat. The supplemented rats gained larger absolute quantities of protein (odds 5000 to 1), but with the deficient rats a higher percentage of the gain of energy was in the form of protein.

The supplemented rats gained an average 6.9 gm. of fat, while the deficient rats gained only 4.0 gm. The significance of this difference is expressed by odds of approximately 1600 to 1.

The addition of the iron and copper compounds to the milk powder unfavorably affected its digestibility. The average digestibility of nitrogen was 91.6% and 88.4%, and of the energy 94.0% and 91.6%, with the deficient and the supplemented rats, respectively. The odds representing the significance of these differences are 3000 to 1 for the digestibility of energy, and over 10,000 to 1 for the digestibility of nitrogen. The urine of the deficient rats, therefore, should contain more energy than should that of the supplemented rats, since the metabolizable energy was essentially the same for both groups, and the digestibility was the higher for the deficient group. This suggestion was found to be correct—the urine of the deficient rats containing an average of 50.8 Calories, while that of the supplemented rats contained 43.6 Calories.

The moisture content of the rat bodies was higher for the deficient rats than for the supplemented rats in eleven pairs among the twelve; the average being 66.0 $\pm$ 0.28% and 63.0 $\pm$ 0.34%, respectively. The significance of this difference is expressed by odds of approximately 4666 to 1. The greater gain of energy by the supplemented rats, therefore, resulted from a greater gain in live weight, containing a higher percentage of dry matter.

From the time the first blood hemoglobin values were determined, 2 weeks after the experiment started, the deficient animals, which received milk powder alone, manifested symptoms of severe nutritional anemia; while the rats which

received the supplements of iron and copper exhibited normal blood hemoglobin values.

The hemoglobin estimations on the blood of the anemic animals were not wholly satisfactory, since the standard colorimeter available at the time a part of these determinations were made was not well adapted to the purpose—the plunger barely touching the liquid, when comparisons were being made with the Newcomer plate.

A micro-colorimeter, designed especially for this type of work, was used for a part of the hemoglobin determinations made at the end of the experiment. Readings were made with both types of colorimeters on the same solutions, and the earlier readings were corrected, by computation, to the basis of the more satisfactory micro-colorimeter determinations. These corrected blood hemoglobin values, therefore, are reported not for a critical study, but only as indicative of the general condition of the rats.

Grams Hb per 100 cc. of blood (average values)

	Fe + Cu deficient rats	*Fe + Cu supplemented rats*
Second week	2.97 ± 0.07	14.28 ± 0.11
Third week	2.93 ± 0.09	14.86 ± 0.19
Sixth week	2.80 ± 0.04	16.10 ± 0.20

The number of blood samples taken from each rat was limited to a minimum, as the taking of the sample occasioned some disturbance to the animal. In such experiments it is desirable to avoid such disturbances, as largely as possible.

When the animals were killed at the end of the experiment, the following observations were made. The skin of the anemic animals was colorless, the hair chalk-white, and the eyes extremely pale. The livers, lungs, hearts and kidneys were also markedly deficient in color. The hearts of all the deficient animals were greatly enlarged; and in ten cases among the twelve, the spleens and kidneys were noticeably larger in the deficient animals. The spleen in the anemic rats was usually enlarged to two or three times the size of the spleen of the normal animals; and the caecum of one of the deficient rats contained evidence of intestinal hemorrhage.

CONCLUSION

In a 6-week growth, metabolism, and body balance experiment, with twenty-four young, growing, albino rats as subjects, an iron-and-copper-deficient diet (whole milk powder) was compared with another diet differing only in that it was supplemented with iron and copper salts. Feeding was controlled by the paired method.

The rats which received the iron-and-copper-deficient diet promptly developed typical cases of nutritional anemia. These rats digested more of the energy and nitrogen of the ration, produced more heat, and stored more water than did those which received the iron and copper supplements, and also limited the food consumption of their normal pair mates.

The rats which received the supplements of iron and copper, made greater gains of body weight, stored more energy, and gained more fat and nitrogen than did the anemic rats, which consumed the same quantities of nitrogen and energy.

The difference between the metabolizable energy values of the diets for the two groups of rats was very small, but the odds that this difference was significant were 100 to 1.

The hearts, spleens and kidneys of the iron-and-copper-deficient animals were generally much enlarged.

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STUDIES ON THE ALLEGED TOXIC ACTION OF COD LIVER OIL

OBSERVATIONS ON GROWTH AND PATHOLOGIC CHANGES IN ANIMALS FED LARGE AMOUNTS OF COD LIVER OIL

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THREE FIGURES

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During the last 12 years there has appeared a long series of papers by Scandinavian workers describing toxic effects following the administration of cod liver oil (Slagsvold, '25; Wahlin, '31; Andersson, '33; Agduhr, '26, '36). Agduhr ('27) working with many species of animals (mice, rats, rabbits, calves, pigs and dogs), claims that pathologic changes may be produced by the oil even when given in daily doses as low as 0.1 cc. per kilogram body weight. Animals of different species were found to differ in their sensitivity to the toxic action of cod liver oil, rabbits and mice being much more susceptible than rats and dogs. The most serious and characteristic lesion was degeneration and fibrosis of the cardiac musculature, although sometimes lesions of the liver, adrenals, kidney and other organs were also noted (Agduhr, '29). In an extensive study of the electrocardiograms of animals in which lesions were produced by the feeding of cod liver oil, Agduhr and Stenström ('29) reported that the conducting tissue was functionally damaged. The toxic factor was found to be associated with the fatty acid fraction of the oil, although in a

[1] Standard Brands, Inc. Fellow, 1934–1936.

later investigation by Agduhr ('36) on the effect of components of cod liver oil upon the tissues of the mouse, lesions in the heart were also observed when the non-saponifiable fraction was fed.

Warning against the injudicious use of cod liver oil has come from a Stockholm clinic. Malmberg ('29) reported two cases of premature infants which were given cod liver oil and showed at autopsy heart lesions similar to those described by Agduhr. One child received 5 to 10 cc. of cod liver oil a day; the other was given 15 to 20 cc. of the oil daily. On the basis of the findings in these two infants, which lived 4 months and 16 days, respectively, and the autopsy reports of which included, in one case acute pneumonia and rickets (craniotabes and internal rachitic rosary), and in the other spina bifida cystica, paralysis of both lower limbs and meningitis, Malmberg states: "It seems that Agduhr's investigations as well as the findings in the cases submitted above . . . . should lead to much greater caution as regards dosage than hitherto has been the case . . . ."

In this country, Madsen, McCay and Maynard ('35) published a series of experiments which strongly suggested that there was a relationship between the ingestion of cod liver oil and skeletal muscle degeneration of Herbivora fed synthetic diets. The heart was also frequently involved. In a later publication Madsen ('36), although he confirmed the previous studies, showed further that cod liver oil was not the only factor concerned in the production of this dietary disease, and that much is left "to be explained as to the causes of the dystrophy which is produced in Herbivora by dietary means."

Cox and Roos ('34) were not able to demonstrate any lesions in rats fed for 130 days a diet in which cod liver oil furnished 78% of the calories. Agduhr's comments ('34) on the negative findings reported by Bell, Gregory and Drummond ('33) would also pertain here: namely, that in spite of the large amount of the oil fed, the experimental period was too short to produce the characteristic lesions in the rat, which at the outset is one of the species least susceptible to the 'toxic' action of cod liver oil.

Evidence has accumulated indicating that diets containing 10 to 15% cod liver oil produce retardation of growth of the albino rat, and that the addition of a liberal amount of yeast to such diets counteracts the unfavorable effect (Harris and Moore, '28; Bell, Gregory and Drummond, '33; De Vries and Puister, '33; Yamamoto, '34). Norris and Church ('30), in their assays of various fish liver oils, also noted this effect of yeast and thought that the toxicity was due to nitrogenous bases (isoamylamine and choline), but Bell and co-workers ('33) found no experimental basis for these conclusions. They demonstrated that the subnormal growth was not due to overdosage of vitamin A or vitamin D; they were unsuccessful in isolating the factor responsible for this effect.

Inasmuch as it is claimed that the mouse is highly susceptible to the toxic effects of cod liver oil (Agduhr and Stenström, '29), the present report deals in part with the effects of feeding large amounts of this oil to mice. In the investigation of the influence of yeast upon the growth of animals fed a high level of cod liver oil, rats were also used, and necropsy findings of those which lived over 150 days are included in the report on pathology.

EXPERIMENTAL PROCEDURE

Diets. The rats were taken at weaning and given a basal diet of constant composition with respect to percentage of foodstuffs; the variant was the type of fat used. The amount of yeast supplement differed among the groups, as discussed in the next section. The constituents of this basal ration were as follows: casein (washed with dilute acetic acid), 24%; rice starch, 45%; fat, 27%, and salt mixture (Osborne and Mendel), 4%. Diet I contained 27% of cod liver oil (Scott and Bowne) which furnished 47% of the total calories. Diet II contained 18% cod liver oil and 9% peanut oil; it furnished 32% of the total calories as cod liver oil. In diet III the source of fat was peanut oil; the ration was supplemented with 40 mg. weekly of a cod liver oil concentrate.[2]

[2] This concentrate was kindly furnished by the Health Products Corporation, Newark, New Jersey. The material had a potency of 72,000 U.S.P. (revised, 1934) units of vitamin A and 9,000 U.S.P. (revised, 1934) units of vitamin D.

The mice were weaned at 21 days of age and received thereafter diets containing 20% by weight of cod liver oil or peanut oil. The amount of yeast included in the rations, however, varied in the two major groups, one containing twice as much yeast as the other.

TABLE 1

Composition of diets for mice

CONSTITUENTS	DIET A	DIET B[1]
	%	%
Casein (86% protein)	25	20
Corn starch	39	34
Fat (cod liver oil or peanut oil)[2]	20	20
Yeast[3]	10	20
Osborne and Mendel salt mixture	5	5
Calcium carbonate[4]	1	1

[1] The total content of protein (which includes that derived from the yeast) is approximately the same in both diets.

[2] Mead Johnson cod liver oil was used and comprised 40% of the total calories. Diets containing peanut oil were supplemented with 40 mg. weekly of a cod liver oil concentrate already described (footnote 2 in text).

[3] Fleischmann yeast no. 2019, having a potency of approximately 15 to 20 Sherman units of vitamin B and 15 to 20 Sherman units of vitamin G.

[4] In order to correct for the large amount of phosphorus in the yeast, the diets were analyzed for calcium and phosphorus, and a calculated amount of calcium carbonate was added to bring the Ca/P ratio to 1.5.

Postmortem examinations and histologic technic. The mice were killed at intervals up to 300 days after the beginning of the experiment, and complete necropsies were performed. The organs of interest, which always included the heart, liver, right kidney and muscle of the right hind leg, were fixed in a solution of formaldehyde, U.S.P. (1:10). Blocks of these tissues were embedded in paraffin and the sections stained with hematoxylin-eosin.

Necropsies were also performed on those rats which were allowed to survive after the completion of the growth studies. The material routinely fixed for microscopic examination was the same as that for mice.

DISCUSSION AND RESULTS

Growth studies. Within each group of rats receiving one of the diets described in the preceding section the amount of yeast supplement varied. Thus, in the groups of rats fed diets I, II and III, respectively, eight of the sixteen animals in the group were given daily 400 mg. of yeast and the remaining eight were given 800 mg. of yeast. The composite growth curves for these groups are presented in figure 1 (B and C).

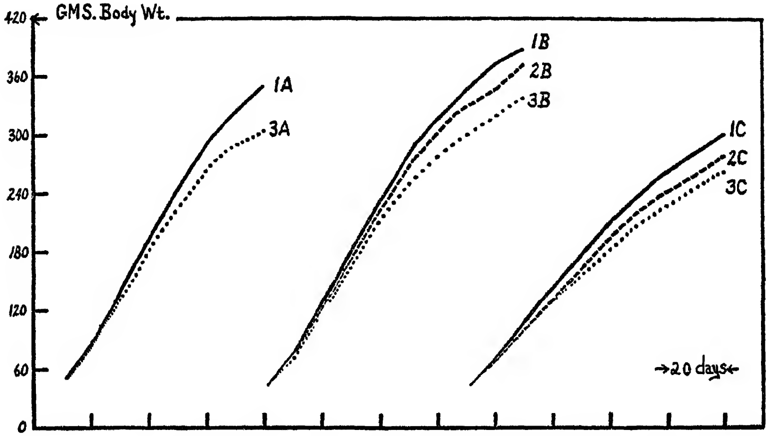

Fig. 1 Composite growth curves of male rats, showing that the differences in growth between animals receiving peanut oil and cod liver oil, respectively, are not affected by varying the amount of yeast supplement. The dotted lines (3A, 3B, 3C) represent the groups which were fed the ration containing 27% cod liver oil (diet I of text). The interrupted lines (2B, 2C) designate the growth of animals given the 18% cod liver oil diet (diet II). The continuous lines (1A, 1B, 1C) represent the control animals given the ration containing peanut oil (diet III). C, 400 mg. yeast. B, 800 mg. yeast. A, 600 mg. of a yeast differing in potency from that given to the other groups.

It is seen that the control animals in each group grew better than the rats receiving cod liver oil, and that the animals consuming the larger amount of cod liver oil grew the least. It becomes at once apparent, however, that no protective action can be assigned to yeast when given in the larger quantity, for although it is obvious that the growth responses in the groups subsisting on the cod liver oil diets were definitely

better when the yeast supplement was liberal, the growth of the control animals was improved to an equal degree.

Another group of animals (twenty-five in all, of which eleven were controls) received as supplement a Fleischmann yeast which was calculated to furnish not much more vitamin B but roughly twice the amount of vitamin G yielded by 400 mg. of the other yeast. This preliminary experiment was undertaken because of the suggestion arising from the work of De Vries and Puister ('33) and Bell, Gregory and Drummond ('33) that the harmful effect of 15% cod liver oil diets might be

TABLE 2

A comparison of the mean gains in weight and food intake of groups of rats fed diets containing cod liver oil and peanut oil

NUMBER OF RATS	DIETARY FAT	YEAST SUPPLEMENT	MEAN GAIN IN WEIGHT	PROBABLE ERROR	SIGNIFICANCE RATIO	TOTAL FOOD CALORIES[1]		
						Mean	Probable error	Significance ratio
		mg.	*gm.*					
8	27% CLO	400	217	10.0	3.8	3310	64	3.4
8	27% PO	400	258	4.4		3620	65	
8	27% CLO	800	293	9.0	3.2	4000	95	4.7
8	27% PO	800	344	13.5		4640	97	
14	27% CLO	600[2]	257	3.8	6.9	3000	32	7.0
11	27% PO	600[2]	301	5.2		3380	44	

[1] Includes those derived from the yeast.

[2] This was a different yeast. The experimental period for this group was 20 days shorter than for the preceding groups.

prevented by a liberal intake of vitamin G. The composite curves for these groups are also presented in figure 1 (A) and show that the difference in growth between the groups receiving cod liver oil and peanut oil is still apparent.

In order to ascertain whether these differences in body weight between the controls and their experimental mates were significant, and whether they could be correlated with the food consumption, the data were submitted to statistical analysis. A summary of the results from animals whose dietary fat was either entirely cod liver oil or peanut oil is given in table 2.

On comparing the significance ratios between the mean gains in weight and mean calorie intake, it is seen at once that the control animals grew better because they consumed significantly more food. With respect to its effect on growth of the albino rat, therefore, no direct toxic action was found attributable to the cod liver oil when given at 18% and 27% levels in the diet.

Observations on the growth of mice were incidental to the major study, i.e., the examination of the histologic preparations of mice killed at various periods. The interpretation of the composite curves presented in figure 2 is therefore complicated not only by discrepancies in the number of animals used in each group but also by the progressively diminishing numbers as the experimental period became greater. Nevertheless, it is apparent that 1) the animals subsisting on the diets containing cod liver oil did not grow as well as those fed rations in which peanut oil was used as the dietary fat, and 2) the growth was, in general, not improved by the addition of yeast in double the amount.

The question at once arose as to whether the differences in weight could be explained on the basis of differences in food consumption. It was very difficult to obtain accurate data on food consumption throughout the entire experimental period, as the mice spilled and scattered their food frequently. An average daily food intake was therefore calculated from a consideration of those periods in which the food could be measured with some degree of accuracy, assuming that the mice ate the same amount of food during the days when the quantity eaten could not be determined. On this basis it was calculated that the gains in weight made by the controls were approximately from 9 to 18% greater than could be accounted for by the differences in calorie intake.

It appears that cod liver oil at a level of 20% in the diet exerts an unfavorable effect upon the growth of the albino mouse. This confirms the statements of Agduhr ('27) that mice are more susceptible to the 'toxic' effects of cod liver oil than rats. However, it may be pertinent to point out that on

the basis of dosage per kilo body weight, the mice received significantly more cod liver oil than did the rats. Thus, as calculated from the daily food intake the dose for mice ranged between 20 to 22 cc. per kilo body weight per day. A similar calculation indicated that the average daily dose for the rat

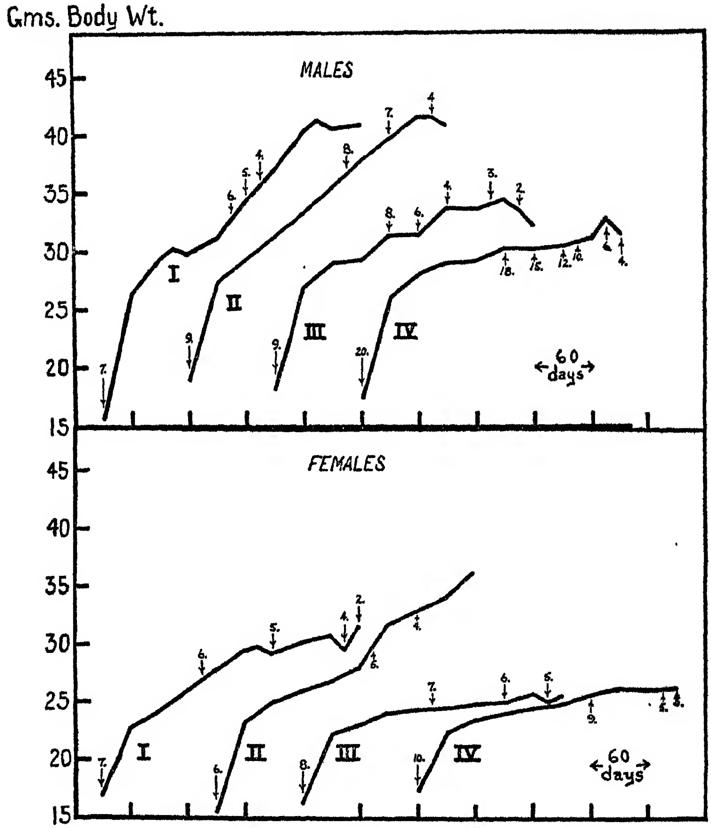

Fig. 2 Composite growth curves of mice representing the males and females of one of the strains used, showing a retardation of growth in the animals which consumed the diets containing cod liver oil. The number of animals progressively diminished because members of each group were killed at various intervals; the figures along the curves indicate the number of animals which furnished the data for the averages during the particular periods of the experiment. The levels of fat and yeast in the diets differed among the groups. These are designated as follows: I, 20% peanut oil and 20% yeast. II, 20% peanut oil and 10% yeast. III, 20% cod liver oil and 20% yeast. IV, 20% cod liver oil and 10% yeast.

was about 11 to 12 cc. per kilo body weight. It would not be profitable, therefore, to draw any conclusions from these data with regard to species differences.

Pathologic findings. Group I. Mice subsisting on a diet containing 20% cod liver oil and 10% yeast. This group included forty-two animals, thirty-four of which lived at least 150 days, and eight of which lived 300 days from the beginning of the experiment. The mice were killed at intervals and routine examination was made of the heart, liver, kidney and skeletal muscle.

Heart. Of the forty-two animals, five showed some degree of fibrosis, four of necrosis, and thirteen in all had variable amounts of pigment-containing mononuclear phagocytes in the myocardium (fig. 3, A and B).

Liver. Ten animals showed deposition of pigment in the liver in variable amount (fig. 3, C and D). In addition, four mice had focal accumulations of small mononuclear phagocytes in the liver.

Kidney. There were no findings of major interest in the kidney. Two animals showed lymphocytic accumulations in the renal cortex. The "calcareous incrustation in the region between cortex and medulla" as described by Åndersson ('33) was not seen.

Skeletal muscle. In contrast to the extensive degeneration of voluntary muscle in herbivorous animals when these are given synthetic diets containing cod liver oil (Madsen, McCay and Maynard, '35), only six animals showed any changes in the muscle of the right hind leg. These changes consisted of an increase in sarcolemmal cells; in addition, muscle atrophy was seen in two animals (living 250 and 294 days, respectively, on the experimental régime). It is of interest that one mouse, which had yellow pigment deposits in both ventricles of the heart, had also an infiltration of pigment-bearing mononuclear phagocytes among the muscle fibers of the leg.

Spleen. Twelve animals had large spleens at necropsy. Microscopic sections of some of these showed the splenic cells to be loaded with finely divided fat. Numerous nests of large

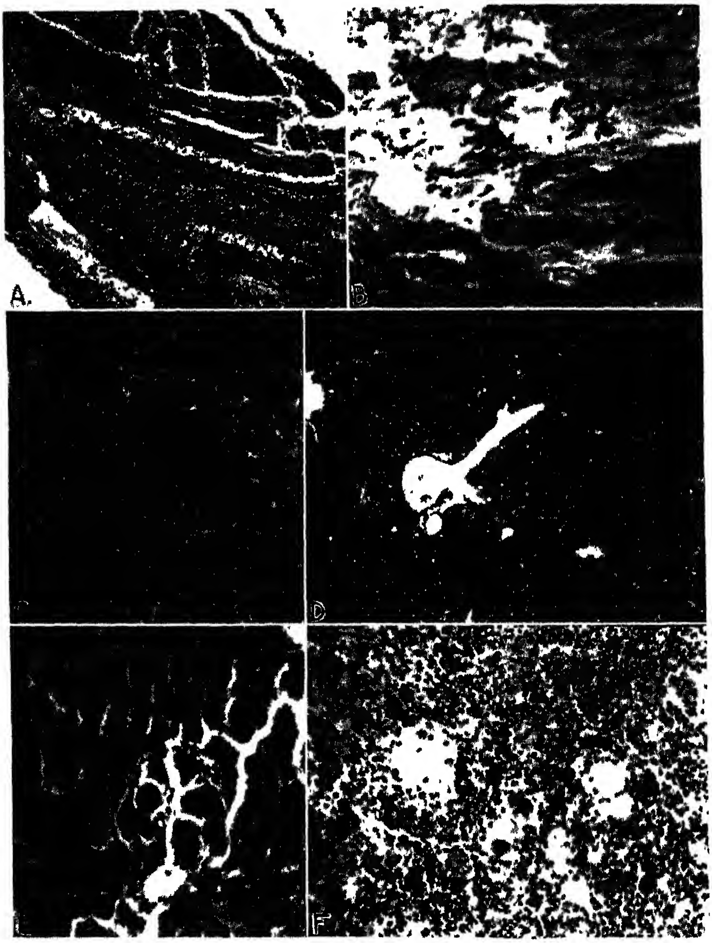

Fig. 3 A, mouse no. 72, group I. Acute and organizing myocarditis of right ventricle. H and E; × 125. B, mouse no. 84, group I. Monocytes containing brown pigment in scarred right ventricular myocardium. H and E; × 200. C, mouse no. 117, group I. Round cluster of liver cells containing brown, lipoid pigment. H and E; × 200. D, mouse no. 77, group I. Calcium salt deposits at sites of lipoid pigment in liver cells. H and E; × 125. E, mouse no. 67, group III. Multinucleated giant cells filled with lipoid pigment in liver. H and E; × 250. F, mouse no. 56, group I. Phagocytosed lipoidal material in mononuclear and multinucleated cells in spleen. H and E; × 200.

mononuclear cells were seen with phagocytosed lipoidal material. Also present were many multinucleated giant cells (fig. 3, F).

Group II. Mice subsisting on a diet containing 20% peanut oil and 10% yeast. Seventeen mice served as controls and were killed in groups with their experimental mates (group I). Of these seven animals lived 300 days after the beginning of the experiment.

Heart. No lesions were found in any of the animals.

Liver. Two of the animals which lived 300 days showed occasional yellow pigment deposits in the liver. Incidental findings were three cases of focal hepatitis, one of pericholangitis and one of a poorly circumscribed abscess.

Kidney. Focal lymphocytic infiltrations in the renal cortex were present in three animals.

Skeletal muscle. All the preparations showed normal architecture.

Group III. Mice subsisting on a diet containing 20% cod liver oil and 20% yeast. This group was composed of nineteen animals. All but one lived at least 150 days, and six were sacrificed after 300 days on the experimental régime. The procedure was the same as that described for group I.

Heart. Three of the nineteen animals showed myocarditis or myocardial fibrosis of mild degree. Four mice had pigment-bearing phagocytes in the myocardium.

Liver. Four animals showed deposition of pigment in the liver in variable amount (fig. 3, E). The livers of two animals contained occasional nests of small mononuclear phagocytes.

Kidney. Four animals had focal mononuclear cell infiltrations in the renal cortex. An incidental finding was a case of marked hyalinization of the glomeruli in an animal which had generalized subcutaneous edema at necropsy.

Muscle. In four mice there was evidence of proliferation of sarcolemmal nuclei, with some atrophy of the muscle fibers.

Spleen. Eleven animals had large spleens at necropsy. Examination of slides from the spleen of mouse no. 41 (see notes under heart and liver) gave a picture of enormous mononuclear cells loaded with lipoidal material. Present also was

a zone of necrosis in which calcium salts were deposited in the fatty material. There was actual bone formation in one zone.

Group IV. Mice subsisting on a diet containing 20% peanut oil and 20% yeast. Seventeen animals served as controls and were sacrificed in groups with their experimental mates (group III). Of these, six animals lived 300 days after the beginning of the experiment.

Heart. No lesions were found in any of the animals.

Liver. The liver of one animal contained a small spheroid zone of calcification (fig. 3, D). Other findings of incidental interest were two cases of focal hepatitis and one of hemangioma.

Kidney. In three animals there were focal lymphocytic infiltrations in the renal cortex. In one mouse a marked hyalinization of the glomeruli was noted.

Muscle. No changes were observed in the skeletal muscles of the right hind leg.

Group V. Rats subsisting on diets containing 27% fat. Twenty-seven rats were allowed to survive after the growth studies were completed. It was hoped to sacrifice them at the end of a year, but some of the animals had secondary infections, and these along with others in the group were necessarily killed earlier. Although the same tissues were studied as those of the mice, the only findings of interest were in the heart.

Five of the eighteen rats fed the diet containing cod liver oil showed necrosis and scar tissue formation in the myocardium, and in two of these, large mononuclear phagocytes were seen containing lipoidal material. It is to be noted, however, that three of these animals had acute infections elsewhere in the body.

Of nine animals serving as controls and subsisting on the diet containing peanut oil, three showed some fibrosis of the myocardium. Of these, one had an acute infection in the lungs.

It is difficult to interpret the results obtained in the rats, as three of the five animals in the cod liver oil series having heart lesions also had concurrent infection elsewhere. Furthermore, three of the controls had fibrotic changes in the myocardium, and of these one had an acute pulmonary infection. It is pertinent to point out that not one of the four animals which subsisted on the 27% cod liver oil diet for 350 days showed any pathologic changes.

COMMENT

Of fifty-two mice which subsisted on 20% cod liver oil diets for at least 150 days, fourteen animals showed in the heart necrosis, fibrosis and accumulations of monocytes with phagocytosed lipoidal material or pigment; of these only four showed as extensive lesions as are described by Agduhr (group I, nos. 72, 84, 33, 61). In the liver, fourteen animals showed changes, of which only six were significant; these were referable to degeneration of hepatic cells, the presence of pigment, multinucleated giant cells and mononuclear phagocytes, and to calcium salt deposition. Twenty-three of the mice had large spleens at necropsy. Unfortunately only a few microscopic preparations were studied, and these indicated fatty deposition of the parenchymal cells, active phagocytosis of lipoidal material by large mononuclear cells, the presence of multinucleated giant cells, necrosis and calcium salt deposition.

That the toxicity of cod liver oil must be considered apart from hypervitaminosis D has been discussed by Cox and Roos ('34). The fact that no calcium deposition was seen in the kidneys of the animals studied also makes it improbable that the changes were related to the intake of vitamin D; it has been pointed out by Steck and co-workers ('37) that the kidney is the most vulnerable of any tissue to the calcifying action of this vitamin.

It is not surprising that the feeding of cod liver oil to mice in a dose approximately 20 to 22 cc. per kilogram body weight per day should produce the effects described, in view of what

is known about the irritating action of cod liver oil. Pinkerton ('28), in studying the effect of intratracheal injection of oils, found that whereas olive oil did not appear to injure the lung in any way, cod liver oil produced a severe inflammatory reaction in the pulmonary parenchyma. Microscopically the alveoli contained enormous, bizarre-shaped giant cells and many large mononuclear phagocytes. Koehne and Mendel ('29) noted that when they administered cod liver oil parenterally the reaction at the site of injection was one of tissue necrosis, giant cell formation and elaboration of young capillaries. This was not seen with any other oil which they used. They make the comment:

> That the oil when so given has toxic properties is evident from the marked deterioration of the physical condition of all animals used and from the marked increases in nitrogen losses resulting in the dog. The body cells treat the injected oil or its emulsion as they would any irritating foreign substance, through the protective reticulo-endothelial system.

More recently Davson ('36) has shown that the favorable results which have been described to follow application of cod liver oil to wounds are not due to the presence of vitamin A (as has been suggested) but to the action of the oil in stimulating the production of granulation tissue.

The same type of proliferative reaction is apparently seen in some animals when large amounts of cod liver oil are ingested over a long period of time. It must be pointed out that in the present study cod liver oil furnished 40% of the calories consumed by the mice (32% and 47% of the calorie intake of the rats). Inasmuch as cod liver oil has been shown to be practically completely absorbed (Agduhr and Stenström, '30; Bell, Gregory and Drummond, '33), the constituents of the oil must have been present in the blood in high concentration during a relatively long experimental period. Under such conditions one might reasonably expect the pathologic changes to have occurred in a much greater percentage of animals than was actually found. In view of this, it is doubtful whether the feeding of amounts of cod liver oil near the therapeutic range would produce any observable injury.

It is of interest that no fatty livers were observed in any of the animals which were consuming large quantities of cod liver oil. The explanation undoubtedly lies in the fact that any effect which the cholesterol in the cod liver oil might have had in producing fatty livers was offset by the choline present in the yeast (Fletcher, Best and Solandt, '35) and the lipotropic factor contained in the casein (Best, Grant and Ridout, '36).

SUMMARY

Rats ingesting diets which contained 18% and 27% by weight of cod liver oil grew at a slower rate than animals receiving peanut oil as the dietary fat. These differences in growth, which were apparent even when the intake of yeast was liberal, were related to differences in caloric intake.

Mice given 20% cod liver oil as the sole source of fat did not grow as well as control animals receiving peanut oil. The presence of 20% yeast in the diet failed to exert any beneficial effect. In this species there was no correlation between body weights and food consumption.

Histologic examination was made of the heart, liver, kidney and skeletal muscle of animals fed diets containing either cod liver oil or peanut oil and sacrificed at various periods on the experimental régime. Some of the animals in the cod liver oil series showed pathologic changes in the heart, liver and spleen consisting of necrosis, fibrosis and phagocytosis of lipoidal material or pigment by monocytes and giant cells. These changes were not seen in any of the thirty-four mice serving as controls for this species, although among the rats three of the controls showed some fibrosis in the heart. It is pointed out that in view of the small percentage of animals which yielded changes in the organs as a result of cod liver oil feeding at high levels, the claims that cod liver oil in therapeutic doses can exert injurious effects are not substantiated.

Grateful acknowledgment is made to Prof. George R. Cowgill for his help and continued interest. It is a pleasure to acknowledge our indebtedness to Dr. Grover F. Powers of the

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METABOLISM STUDIES WITH RATS SUFFERING FROM FAT DEFICIENCY [1]

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SIX FIGURES

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INTRODUCTION

Rats suffering from fat deficiency (Burr and Burr, '29) show the peculiarity of consuming as much food as normal rats, and yet remaining small and finally becoming emaciated (Burr and Burr, '30). Furthermore, the deficiency symptoms are cleared up by the addition of small amounts of certain fats or fatty acids, and the rats resume growth. This raises a question as to the ability of these rats to synthesize fat, especially the essential fatty acids.

Wesson and Burr ('31) showed that following a carbohydrate meal the respiratory quotients of fat-deficient rats were well above unity, indicating ready synthesis of fat. The basal and assimilatory rates in the case of young rats in the early stages of fat deficiency were well above normal. This readily explained why the rats required the usual quantity of food although they were much smaller than the normal controls. Wesson and Murrell ('31) have extended this work and have shown a curative effect of the liquid fatty acid fraction of lard.

[1] Supported by grants from the medical research fund and general research fund of the University of Minnesota and from the National Live Stock and Meat Board.

For the above metabolism studies the rats were starved 14 hours and then fed sufficient dextrin for 12 hours of normal metabolism. This meant the ingestion of a large quantity of readily absorbed food in a few minutes, and although it gave an excellent comparison of the different rats, there was some doubt as to whether this procedure gave an insight into the normal behavior of the animals. The following experiments were designed to show how the rats differed from the normals when they were in their usual cage and on their regular diet.

APPARATUS

Since it was desirable to continue the studies for 24 to 48 hours, an open circuit apparatus was constructed. A constant speed, constant temperature spirometer drew a stream of dried outside air through the rat chamber. As the air left the chamber it was dried over sulfuric acid and calcium chloride. It then passed by a sampler which continuously aliquoted the air stream, and held the dry gas over dry mercury until time for analysis. The sampler is shown in figure 1. All the collecting vessels are full of mercury at the beginning of the run. The flasks are lowered by the constant speed windlass at any desired speed so that a sample may be collected in a few minutes or in several hours. The first collector begins after a preliminary period of about 30 minutes. Just as the first finishes the second begins to collect. All of the mercury receiving flasks are being lowered all the time, but the capillary tubes are so small as compared with the larger sample holder that 99% of the sample is collected as the flask passes the sample holder and only 1% collected in the capillary. After all samples are collected a correction may be applied, but it is too small to be considered in this work. The air passes over a capillary mercury trap which prevents any back diffusion of gases.

The collecting vessels hold about 125 cc. and are 4 inches long. The speed of the windlass was regulated for this work so that a sample was collected in 4 hours. Therefore, six collectors covered a period of 24 hours. Since these samples

are continuously aliquoted they represent the mean gas exchange for each 4-hour period and do not have the uncertainty in composition which is characteristic of an instantaneously collected sample.

The samples and spirometer are driven by the same shaft. The temperature of both remains the same and is constant to 1°C. Both get the same effect from barometric changes.

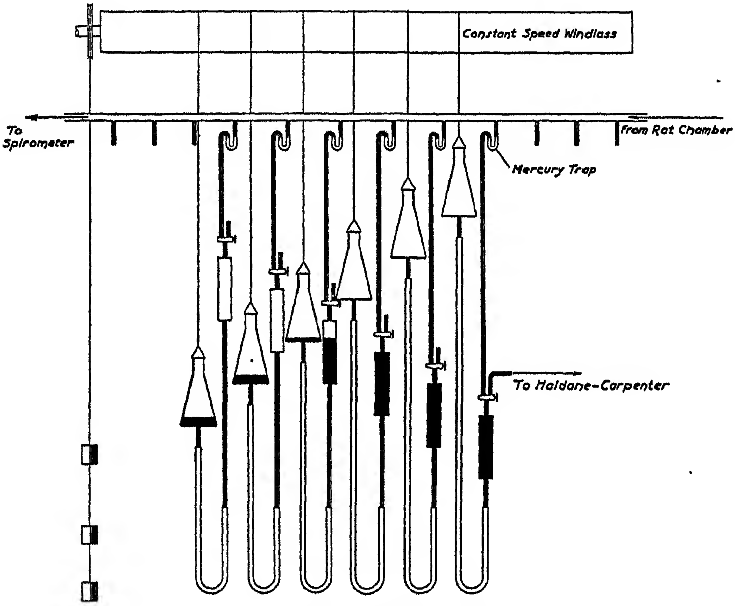

Fig. 1 A sampler which continuously aliquots the gas stream passing from the rat chamber to the spirometer.

Therefore, the same percentage of the air stream is being aliquoted at all times.

A chain of lead weights rises and counterbalances the mercury as it flows into the flasks. This keeps the windlass from becoming too heavily weighted on one side.

When it is time to begin the analyses the stopcocks are closed and the mercury flasks are hung on hooks so that the gas is under a slight positive pressure. A capillary tube leads

directly into an extra three-way capillary stopcock which is fastened to the Haldane-Carpenter. The tubes are washed out with the sample and the analyses for CO_2 and O_2 carried out according to Carpenter's (Carpenter, Fox and Sereque, '29) directions, all precautions being observed.

The spirometer has been set at such a speed that the maximum CO_2 is 1.6 volume per cent and most of the O_2 and CO_2 values remain above 1%. The Haldane-Carpenter has proved highly satisfactory, permitting an accuracy of 0.005% on the O_2 and 0.002% on the CO_2. Therefore, the second decimal of the R.Q. is probably correct.

The spirometer temperature remained constant within a range of 1°C. The barometric pressure and temperature being known the volume of air (at 0° and 760 mm.) passing through the chamber was determined, and from this the metabolic rate was calculated. The maximum fluctuation in speed of the spirometer is 1.5%. The rates probably are accurate within 2%. For quotients above unity the calorie value of oxygen at an R.Q. of 1.00 is used, with no correction for the excess CO_2. No nitrogen correction is made.

The colony temperature is maintained at 25.5°C. ± 1° the year round. All rats used in this work were acclimated to this temperature.

The metabolism chamber of thin metal was immersed in a water bath kept at 28.5° and the temperature inside the chamber was approximately the same. The change in temperature encountered by the rat on entering the metabolism chamber from the colony room was only 3°C.

PROCEDURE

All experimental rats were fed their daily supplements (vitamins, oils, etc.) about 4 o'clock each afternoon. Therefore this was considered a good time to start runs showing normal behavior. Three different studies were made. The results are summarized in figures 2, 3 and 4 and table 1 (A, B and C).

TABLE 1

Calories per individual rat are given for each 4-hour period. These calculations are independent of size.

PERIOD	1	2	3	4	5	6	7	8	9	10	11	12
A. Rats in figure 2												
Fat cures (average)	3.70	3.91	5.07	5.57	5.00	4.71						
Stock rats (average)	3.75	4.00	3.98	4.31	3.57	3.55						
Fat-starved (average)	4.01	4.1	5.51	5.68	4.78	4.77						
B. Rats in figure 3												
Fat cures (average)	5.31	5.70	5.17	4.67	3.87	3.63	3.95	4.51	4.50	4.21	3.80	3.61
Stock rats (average)	4.30	4.81	4.78	4.31	4.08	4.28	3.65	3.97	3.90	3.90	3.61	3.30
Fat-starved (average)	6.07	6.20	5.60	5.13	4.40	4.15	4.15	4.30	4.57	4.43	4.21	3.73
C. Rats in figure 4												
Stock rats (average)	4.31	4.68	4.88	4.57	3.93	3.60	4.55	5.40	5.33	4.51	4.03	4.07
Fat-starved (average)	6.13	6.27	5.75	5.10	4.35	4.78	5.60	6.10	5.35	5.28	4.61	4.18

One group (fig. 2) was starved 16 hours in the colony, transferred to the metabolism cage, and two samples were collected during the next 8 hours of fasting. The chamber was opened at 4 P.M. and the rats fed their yeast doses and other supplements, weighed cups of their usual diet put in

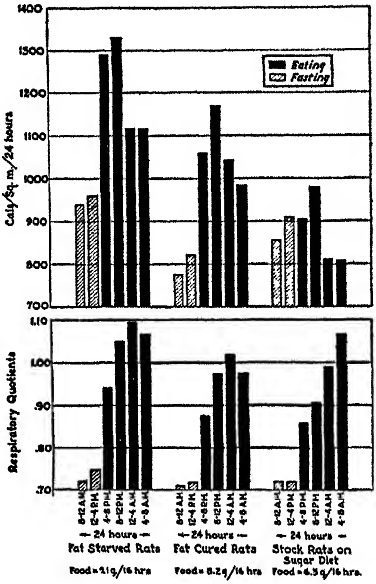

Fig. 2 Metabolic rates and respiratory quotients of three types of rats. Each column gives the mean value for a 4-hour period. Total time of each run is 24 hours. Rats were starved in colony cages 16 hours, transferred to the metabolism chamber and fasting values recorded for two periods (8 hours). The yeast dose and food were then put in the chamber and the values while eating were recorded for 16 hours.

and the run continued. A water bottle was always in the cage. The stock rats in this group were fed, during the run, the pure diet[2] mixed with 7% Northwestern dried yeast.

The next group (fig. 3) was not starved at all before the run. They were fed their supplements at the usual time (about 4 P.M.), immediately put into the chamber with their

[2] The basal fat-free diet fed in all this work consisted of: pure casein 12.0%; sucrose 84.1% and salts mixture 3.9%. This was supplemented by 0.7 gm. dried yeast and concentrates of vitamins A, D and E (Burr and Burr, '29).

usual diet and the gas exchange followed for 24 hours (six samples). Then the food cup was removed and the metabolism followed during 24 hours of starvation. Again, the stock rats were fed the pure diet plus 7% yeast instead of their usual stock diet.

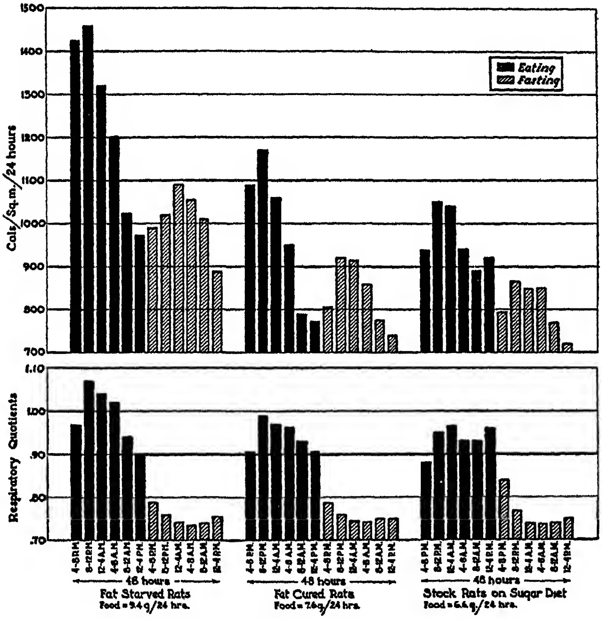

Fig. 3 Metabolic rates and respiratory quotients of three types of rats. Each column gives the mean value for 4 hours. Total time of run 48 hours. Food was in the cage during the first 24 hours (black column). Food was removed during the next 24 hours (shaded columns).

The third group (fig. 4) was studied for 48 hours without any starvation. Sufficient food for 48 hours was put in the cage. After the first 24 hours the chamber was opened, the supplements were fed and the run continued. In this case the stock rats were fed their usual stock diet. However, the chamber was opened and the food consumption measured at the end of 24 hours so that they were disturbed in exactly the same way that the fat-starved rats were.

Wesson and Burr ('31) noted that there was no greater activity by the fat-starved than by the other rats. The increased metabolic rate was shown by sleeping rats. However, some records of activity were made in connection with the present work. A typical 48-hour record is shown in figure 5. The activity at night is followed by long periods of rest during the day. On the whole, the records do not show that one type of rat was more active than another.

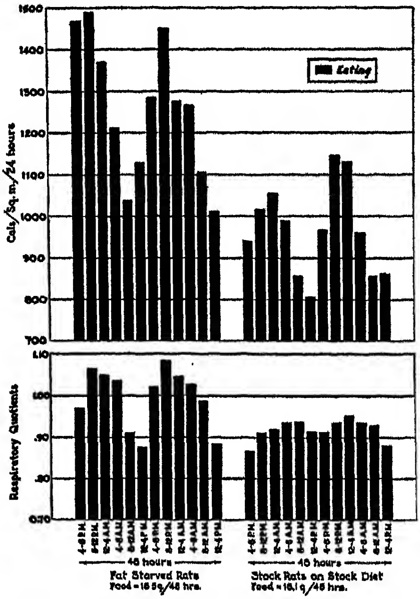

Fig. 4 Metabolic rates and respiratory quotients of fat-deficient and stock rats. The runs were continued 48 hours with food in the cage the entire time. The fat-deficient rats received the purified diet and the stock rats the usual stock diet.

Each line across the graph represents 2 hours, 15 minutes in time.

The records are made by suspending the cage on a spring so that each movement has a definite value in grams. The cage is built of extremely light material with a total weight less than that of the rat. This decreases the inertia and gives to the recorder a high sensitivity. The marks about 1 mm.

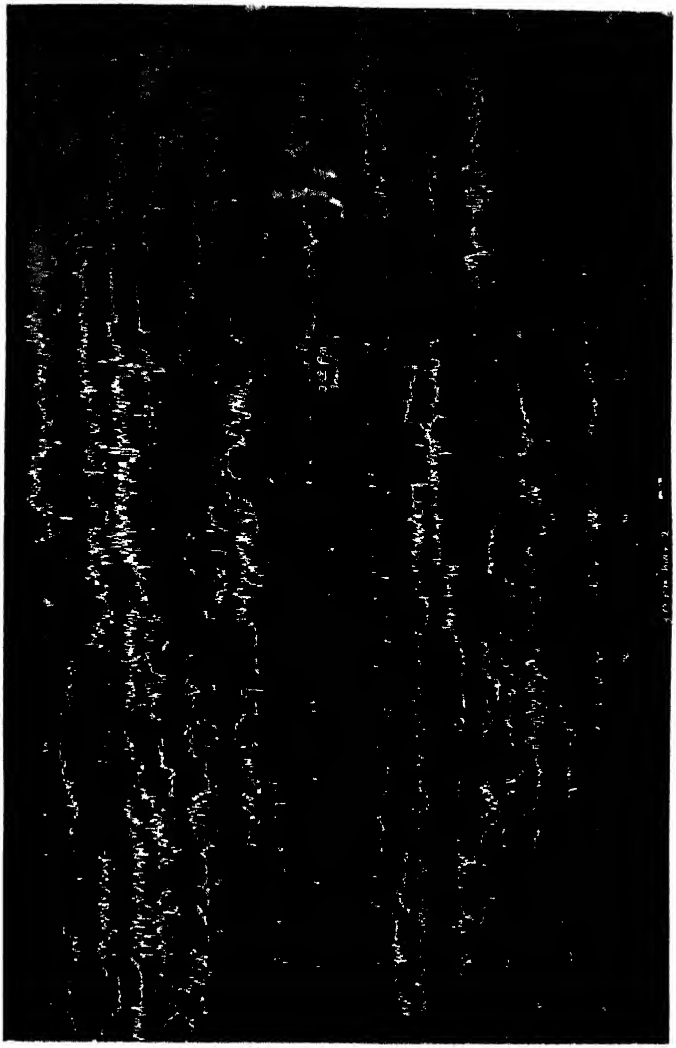

Fig. 5 Forty-eight-hour activity record of a fat-deficient rat. Food was in the cage throughout the run.

long at intervals in periods of rest are made by a slight movement of the head.

In this work the single formula of Lee and Clark ('29) was uesd for calculation of surface area ($S = 12.54 \times W^{0.60}$). As pointed out by Brody ('34) methods of measurement of surface are not satisfactory and heat production per unit of surface has no absolute meaning. However, the values given in figures 2, 3 and 4 are useful for comparison with data in the literature. The absolute calorie production by each rat over a period of 4 hours is given in table 1 (A, B and C).

RESULTS

The columns in figures 2, 3 and 4 give the average values of from four to eleven runs on as many different rats. The average food consumption per rat for the period during which food was in the chamber is given below each group. It was shown earlier (Burr and Burr, '30) that rats cured with fat consume the same amount of food as the fat-starved rats. But when these rats are put in the metabolism chamber at the higher temperature there is a tendency for the fat-starved rats to continue their usual consumption of food while the cures eat much less than normally. This is reflected in the high metabolic rates and high R.Q.'s. The high quotients and high metabolic rates reported by Wesson and Burr ('31) are confirmed. The addition of 10 drops daily of an oil has made the rats (fat cures) behave much more like the stock rats both with respect to quotient and rate of gas exchange. It is very interesting to see that when food is available the fat-starved rats maintain an average quotient above unity for as much as 16 hours out of 24 and in the second day of figure 4 this continued for 20 hours. If the high quotients are given the usual interpretation, i.e., fat synthesis, then it must be concluded that fat-starved rats readily synthesize fat. As has been pointed out in our earlier papers, analytical evidence as well as the curative effect of methyl linolate or linolenate indicate a very limited ability to synthesize these essential fatty acids. It would seem, therefore, that fat

synthesis from carbohydrate is largely confined to the more saturated acids.

The daily cycle of activity of all the groups is clearly shown in figures 3 and 4. The first column represents the period from 4 to 8 P.M. The metabolic rate reaches a peak from 8 P.M. to 4 A.M. and then falls to a low level during the day. This happens even during starvation as shown by the second day of figure 3. It is interesting that this is not associated with light since the chamber is almost totally dark.

There are some other facts of general interest in the field of metabolism. Periods of starvation of from 12 to 17 hours have been recommended in the study of basal metabolism (Benedict and MacLeod, '29). The starvation records in figure 3 show that a peak of metabolic rate is reached in 12 to 16 hours in all groups. Possibly this is due solely to increased activity because of hunger or because it is the middle of the night. The same curves should be run again with the starvation begun at another time of day. However, this metabolic curve as it stands indicates that the best chance of obtaining a short period basal on rats comes just after noon after a 24-hour starvation.

The R.Q.'s very uniformly reach a minimum at from 12 to 16 hours starvation during the period of maximum activity (fig. 3). It is interesting that the rats starved 18 hours in the colony and then put in the chamber (fig. 2) usually showed somewhat lower quotients than the rats starved in the metabolism chamber (fig. 3). There is an average difference of about 0.02.

The graphs show clearly that the utilization of carbohydrate as shown by high quotients does not greatly raise the metabolic rate. The stock rats of figure 2 show this most strikingly. They had been starved 18 hours in the colony room and transferred to the chamber. During the next 8 hours the average rates remained between 850 and 900 cals./sq.mm./24 hours. But as soon as they had eaten all they wanted and were asleep again the next day the rates fell to 800 calories (well below the starvation rates) while the quotients rose to values above unity.

On the other hand, the great rise of metabolic rate which occurs immediately after the feeding of the yeast dose at 4 P.M. indicates that the taking of this material into the stomach does greatly increase metabolism. It would seem, therefore, that the early digestive action on certain foods demands a great increase in metabolism while the absorption and utilization of carbohydrates resulting in high R.Q.'s produces no large increase in energy release. An estimate of the effect of food on metabolic rate during different periods of utilization can be made from the data in figure 3 if it is assumed that activity during each period of starvation is the same as for the corresponding period when food was in the

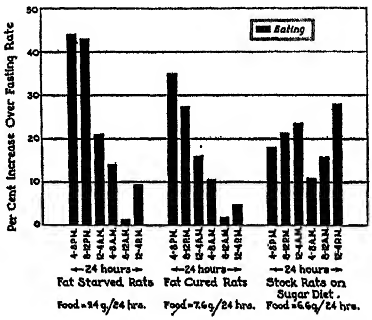

Fig. 6 The effect of food on metabolic rate expressed in percentage increase over the starvation rate. The values were obtained from figure 3 by subtracting column 7 from column 1, column 8 from column 2, etc.

cage. The percentage increase of rate with food in the cage over the rate during starvation is shown graphically for the six periods in figure 6. The fat-starved rats show an increase of 44% soon after receiving their yeast dose. The excess rate has fallen to 14% in the fourth period although the quotient is still well above 1.00. By 8 o'clock in the morning, the increase in rate has fallen to 1% while the quotient is 0.94. These high quotients indicate continued absorption of carbohydrate and probably fat synthesis. It appears, therefore, that the early digestive action on certain foods demands a great increase in metabolism while later utilization may be carried on at a basal level of energy exchange.

This is in accordance with two statements made by Lusk ('23): "It is, therefore, apparent that the intensity of metabolism is not related to the height of respiratory quotient;" and, "It happens frequently that with the cessation of glucose absorption the respiratory quotient remains at 1.00 indicating that carbohydrate is still the essential food, and yet the metabolism has fallen to the basal level."

Recent experiments have led to the view that Lusk's plethora theory as applied to carbohydrates must be abandoned or greatly modified (Wilhelmj, '35). The experiment here presented may be interpreted as giving evidence that the intermediates in the reaction carbohydrate→fat do not have a large S.D.A. Unlike the conversion of glucose to glycogen, the synthesis of fat goes spontaneously as an ordinary fermentation, and this reaction is not accompanied by any increased burning of carbohydrate as evidenced by intake of oxygen.

The very great increase in metabolic rate during the period of greatest food intake (from 4 to 12 P.M.) followed by an essentially basal level while the R.Q. is still high leads to the view that in carbohydrate foods the factors most important in raising the metabolic rate are to be found in water exchange (Carpenter and Fox, '30), work of digestion (Zuntz's Verdauungsarbeit) (Brody, '34) and glycogen synthesis (Wilhelmj, '35).

SUMMARY

Fat-deficient rats may synthesize much fat each day as indicated by high respiratory quotients.

The fat synthesized from carbohydrate does not contain appreciable quantities of the essential fatty acids since these must be added to the diet to prevent decline and death.

Although much smaller, the rats have a higher metabolic rate than their controls. Consequently, they have a much higher rate calculated as calories per square meter of surface.

A normal diurnal activity is shown for all groups, which is independent of light and food.

The minimum R.Q. is reached after 12 to 16 hours of starvation at 28.5°C.

Minimum metabolic rate is reached in the afternoon after 20 hours of starvaton.

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CONTROL FEEDING TECHNIC IN BONE CALCIFICATION STUDIES [1]

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Among the many factors known to affect the calcification of bone, the calcium content of the food has been shown to be of importance. Thus, by incorporating different quantities of this element into the ration Sherman and Booher ('31), Shohl and Wohlbach ('36), Brown et al. ('32), Bethke, Edgington and Kick ('33), Mendel ('34), and Fairbanks and Mitchell ('36) were able to alter the rate at which bone salts were stored by experimental animals. These findings point to the need for eliminating chances of a shifting intake of calcium, and possibly of phosphorus, in studies concerning the calcification of bone. A source of such an error is introduced under conditions of ad libitum feeding if the elements are incorporated into the ration at a constant percentage. This fact has been largely unappreciated, although, in studies in which two diets are being compared, it is not uncommon to find animals on the more satisfactory ration consuming twice as much food as those on the poorer ration. Even in vitamin D assay studies in which the amount of ash deposited in the bones has been suggested as the sole criterion, provision for the control of this factor has not been made (Sherman and Stiebeling, '29; Hart, Kline and Keenan, '31; Griem, '34).

[1] This paper appeared on the program of the annual meeting of the American Home Economics Association, June, 1933.

[2] A portion of these data was presented in partial fulfillment of the requirements for the degree of master of science at the University of Illinois by Lillian Merritt, June, 1932.

It is highly conceivable that much of the lack of uniformity in bone ash values observed by some investigators is due to differences in food consumption (and hence in mineral intake) as fostered by ad libitum feeding technic.

With this possibility in mind the present study was started in 1931. The data demonstrate that, on the ingestion of a ration adequate in all respects and containing a fairly generous percentage of mineral salts, the litter mate animal which consumes the more food will store the more ash in its bones. Two technics are described which have been successful in reducing such variations which might occur as a result of differences in appetite between animals being compared. One method involves the use of control feeding as sponsored by Mitchell ('29–'30), the other, the feeding of the calcium and phosphorus moiety of the mineral salts apart from the ration.

EXPERIMENTAL

The experimental animals were healthy, young albino rats taken from a colony that had been reared and maintained on an apparently satisfactory ration [3] for several years. The litters were reduced in number to eight young on the second day after birth and were taken from the mother on the twenty-first day. Female rats weighing at least 37 gm. at weaning were used. They were confined to individual cages with false bottoms to reduce coprophagy. The amount of light to which they were exposed was reduced to a minimum by placing them in a north basement room and by hanging a closely woven black cloth around the shelves on which the cages were kept.

For 1 week following weaning the rats were on a vitamin D-free ration to aid in the depletion of any possible stores of

[3] The ration for the breeding colony had the following composition: 2400 gm. ground whole wheat, 1200 gm. whole milk powder (Klim), 360 gm. ground meat scrap, 48 gm. C. P. NaCl, and 200 gm. brewers' yeast. Lettuce, in amounts of 40 gm., was given twice a week and the same amount of carrots once a week until parturition at which time they were withdrawn. During the periods of growth and rest between litters one-half of the whole wheat was exposed to ultraviolet light for a period of 30 minutes at a distance of 18 inches. During pregnancy and lactation the ration was not irradiated.

vitamin D. This ration had the following composition: low-ash casein (Swanson, '30) 18, starch 78, and salt mixture no. IV (Osborne and Mendel, '18) 4%. Yeast [4] and spinach powder [5] were fed daily at levels of 400 mg. and 50 mg., respectively.

For the experimental study three litter mate rats, as identical as possible in weight, appetite, and vitality, were selected to comprise a group. All animals of this triad were given the same basal ration plus the allotted supplements with the exception of vitamin D. Two of these animals were then chosen for a paired-feeding regimen in which the animal with the poorest appetite determined the quantity of basal ration consumed by both. One received cod liver oil [6] daily, the other, the same amount of corn oil [7] but no vitamin D. The third rat of the triad was fed the basal ration ad libitum and cod liver oil daily. The animals were kept on these diets for 28 days and hence, were 56 days old when the experiment was terminated.

The criterion adopted for the calcifying effects of the ration was the amount of ash deposited in the bones. The animals were sacrificed with ether and both femurs immediately excised. The technic used in the removal of the tissues and in the desiccation, extraction, and ashing of the bones has been fully described in another publication (Outhouse, Macy, and Brekke, '28). The ash content was calculated on the basis of the fat free, moisture free femur.

Series 1. The ration was essentially that advocated by Sherman and Stiebeling ('29) for the quantitative estimation of vitamin D and consisted of the following: low-ash casein 18, starch 78, and salt mixture no. IV (Osborne and Mendel, '18) 4%. Daily adjuvants for each animal of the triad were 400 mg. of yeast and 50 mg. of spinach. These were fed apart

[4] Dried brewers' yeast purchased from the Northwestern Yeast Company, Chicago.

[5] Powdered spinach prepared by the California Vegetable Concentrates, Inc., Los Angeles, California.

[6] Sample no. 156 of cod liver oil obtained from the E. L. Patch Company, Stoneham, Massachusetts.

[7] The commercial brand, Mazola, was used.

from the ration in order to insure sufficient vitamins A, B, and G for growth, irrespective of the amounts of basal food eaten. Cod liver oil, in a 50 mg. portion, was fed daily to one of the pair receiving identical amounts of the basal ration and to the other member of the triad eating ad libitum.

Series 2. In this series the calcium and phosphorus moiety of the salt mixture was fed separately from the basal ration which consisted of low-ash casein 18, starch 79.4, and salt mixture no. XXX [8] (Osborne and Mendel, '26–'27) 2.6%. The daily adjuvants for each member of the triad were 400 mg. of yeast, 50 mg. of spinach powder, and 287 mg.[9] salt mixture no. IV. As in series 1, two animals of the group were given 50 mg. of cod liver oil daily, whereas the third rat received no vitamin D. In order to insure the ingestion of the mineral salts the dry adjuvants were mixed together with a small portion of the basal ration. The remainder of the ration was withheld until complete consumption of the supplements occurred.

DATA AND DISCUSSION

Series 1. The data for food intake, weight gains, and bone ash for the fifteen triads in this series are presented in table 1. The amount of food eaten by the rats on paired feeding ranged from 134 to 229 gm. for the 28-day period. In each case the animal without added vitamin D had the poorer appetite and hence, limited the amount of food eaten by its pair mate. In every triad the rat on the ad libitum feeding plan consumed considerably more food than did its litter mate on paired feeding; the total increase ranged from 38 to 137 gm.

The weight gains made by the rats receiving no vitamin D were variable with a minimum of 29 and a maximum of 67 gm.

[8] The salt mixture no. XXX is free of calcium and phosphorus; at a level of 2.6% the mixture furnishes the elements other than calcium and phosphorus in approximately the same amounts and proportions as would be supplied by 4% of the Osborne and Mendel salt mixture no. IV.

[9] This quantity of salt mixture contained the same amount of calcium as was found in 30 cc. of whole milk. This level of milk was shown, in a preliminary study, to induce normal calcification in bones of rats fed the ration as used in this series.

for the 28-day period. Their pair mates receiving cod liver oil attained greater weights. The increase was slight; but it appeared in thirteen of the fifteen triads and was found, on analysis of the data (Student, '15–'17), to be statistically

TABLE 1

Response of litter mate groups to the ad libitum ingestion of a mineral-rich basal ration

LITTER MATE NUMBERS	PAIR WITH SAME INTAKE OF BASAL RATION					LITTER MATE FED BASAL RATION AD LIBITUM AND VITAMIN D		
		Rat fed no vitamin D		Rat fed vitamin D[1]				
	Food intake (28 days)	Gain in weight (28 days)	Ash content of bone[2]	Gain in weight (28 days)	Ash content of bone	Food intake (28 days)	Gain in weight (28 days)	Ash content of bone
	gm.	*gm.*	%	*gm.*	%	*gm.*	*gm.*	%
2602–04[3]	207	58	52.3	55	59.4	262	81	60.7
2605–08[3]	181	40	52.8	50	59.6	237	85	60.6
2634,35,39	168	42	51.7	60	55.2	305	80	59.2
2636–38	180	40	47.4	58	55.0	309	80	59.2
2696,97,2701,02[3]	229	53	56.3	60[4]	58.6[4]	295	86	60.1
2698,99,2700[3]	212	60	49.7	65	59.0	275	85	59.5
2802–06	203	54	55.9	67[4]	60.4[4]	254[4]	80[4]	61.1[4]
2807–09	222	67	56.9	67	60.2	260	84	61.4
2810–12	134	29	53.6	37	57.1	266	82	60.3
2813–15	174	46	46.4	53	56.8	248	76	59.7
2872,76,77[3]	173	36	52.2	49	58.4	270	95	60.8
2873,75,78[3]	181	41	50.5	52[4]	57.4[4]	243	83	58.9
2893–95	182	45	49.4	49	58.6	259	84	57.7
4449,50,54	143	39	50.1	41	57.2	224	70	60.9
4497–4500	144	39	50.3	41	56.4	229	75	59.1
Average	182	46	51.7	54	58.0	262	82	60.0
Standard deviation	27.6	10.0	3.0	9.1	1.6	24.7	5.5	1.0
P.E.	18.6	6.7	2.0	6.1	1.1	16.7	3.7	0.7

[1] Fifty milligrams cod liver oil daily.
[2] Calculated on basis of dried, alcohol-ether extracted bone.
[3] Litter mate groups.
[4] Average of two rats.

significant as shown by odds greater than 9999 to 1. The inability of this group to make greater gains was due, in large part, to the restricted food supply. This was evidenced by the fact that the litter mates fed cod liver oil and eating ad libitum grew decidedly better.

The ash content of the bones of the animals on any one of the dietary modifications showed considerable variation as seen in table 1. In comparing the rations, however, significant differences are in evidence, whether the data be considered for each triad or as an average for all the members on a given regimen. As might be expected, in each of the fifteen triads the animal receiving cod liver oil had an appreciably greater bone ash content than had the other member of the pair receiving the same amount of food. The difference between 58.0 and 51.7% ash for the rats receiving cod liver oil and no cod liver oil, respectively, represents an average increase of 12% in the relative amount of ash deposited in the bones. This increase is definitely attributable to the vitamin D in the cod liver oil. Of the two animals receiving this adjuvant, the one consuming the ration ad libitum had the more ash in its bones in fourteen of the fifteen triads. On statistical analysis of the differences, odds greater than 9999 to 1 were found, indicating practical certainty that the results were not fortuitous.

Inasmuch as the only difference between the diets of the two animals receiving cod liver oil was the quantity of basal ration consumed, one may conclude that the increase in bone ash was due to the greater quantity of food ingested by the animal on the ad libitum regimen. This series demonstrates, then, that with mineral rich rations a disparity in the amount of food consumed by animals being compared can bring about a difference in the amount of ash deposited in the bones. With this type of ration, therefore, a plan of control feeding should be instituted in studies in which bone ash values are used as the sole criterion.

Series 2. In this series the possibility of a difference in the rate of calcification being induced in animals as a result of unequal appetites was decreased by feeding the calcium and phosphorus portion of the mineral salts in a constant daily amount separate from the ration. The results for the sixteen litter mate triads are tabulated in table 2. The amount of food eaten by the animals on the paired feeding plan varied

from 112 to 206 gm. for the 28-day period. As in series 1, the rat receiving no cod liver oil had the poorer appetite of the pair and hence, limited the amount of food that its pair mate received. The litter mates on the ad libitum regimen

TABLE 2

Response of litter mate groups to the ad libitum ingestion of a basal ration deficient in calcium and phosphorus

LITTER MATE NUMBERS	PAIR WITH SAME INTAKE OF BASAL RATION					LITTER MATE FED BASAL RATION AD LIBITUM AND VITAMIN D		
	Food intake (28 days)	Rat fed no vitamin D		Rat fed vitamin D[1]				
		Gain in weight (28 days)	Ash content of bone[2]	Gain in weight (28 days)	Ash content of bone	Food intake (28 days)	Gain in weight (28 days)	Ash content of bone
	gm.	*gm.*	%	*gm.*	%	*gm.*	*gm.*	%
3246–48	206	53	54.5	67	58.7	223	82	56.9
3801–05	137	23	55.5	30[3]	59.2[3]	213[3]	69[3]	60.0[3]
3806–09	193	44	52.5	50	57.1	219[3]	70[3]	58.1[3]
3831,33,35[4]	165	33	54.9	36	59.0	242	83	60.1
3832,34,36[4]	167	47	53.9	47	58.5	232	81	59.9
3826–29	173	30	57.1	29	61.7	248[3]	80[3]	60.5[3]
3837–40	175	36	57.6	58	61.2	203[3]	88[3]	59.0[3]
3862–64	161	46	53.2	57	60.0	232	78	60.1
3867–69	161	44	53.2	45	58.2	232	82	58.5
4393–95	134	36	54.2	35	58.8	226	81	59.5
4396–98	141	31	51.4	34	58.8	243	80	58.1
4387,90,91[4]	112	20	52.4	20	57.6	215	75	59.3
4388,89,92[4]	112	15	52.4	12	59.1	193	54	60.2
4384–86	129	31	50.9	31	57.8	236	82	58.8
4408–10	117	31	52.8	35	56.4	191	82	57.8
4498–4502	112	21	50.9	20	55.1	244	77	55.4
Average	150	34	53.6	38	58.6	225	78	58.9
Standard deviation	28.8	10.5	1.9	14.6	1.6	17.2	7.7	1.3
P.E.	19.4	7.1	1.3	9.8	1.1	11.6	5.2	0.9

[1] Fifty milligrams cod liver oil daily.
[2] Calculated on basis of dried, alcohol-ether extracted bone.
[3] Average of two rats.
[4] Litter mate groups.

in all cases had greater avidity for food. In general, however, the food intakes were appreciably lower than in series 1, in which 73% of the animals consumed 248 gm. or more of food. None of the rats in series 2 partook of more than this

amount. The cause of this poor food intake was not determined. It was appreciated during the experiment, however, that the animals did not particularly relish the mixture of adjuvants containing the inorganic salts. Inasmuch as the basal ration was withheld largely until the supplements had been consumed, it is possible that the animals did not have sufficient time in which to obtain an adequate amount of the basal ration.

The gains in weight made by the animals without cod liver oil were not very satisfactory, averaging 33.8 gm. for the 28-day period. The addition of cod liver oil to the ration stimulated growth above that in the pair mate in nine of the sixteen cases. The chances that this increase was not accidental were found on statistical analysis to be 57 to 1. The member of the triads eating ad libitum and receiving cod liver oil, however, gained, as an average, more than twice as much as either of the paired animals. The magnitude of their gains was similar to that observed for series 1.

The relative amount of ash laid down in the bones of the animals receiving no cod liver oil showed a range from 50.9 to 57.6% and averaged 53.6. In every one of the sixteen cases the feeding of vitamin D to the pair mate resulted in a definitely greater amount of bone ash—the data ranging from 55.1 to 61.7%. The average value of 58.6% represents an increase in bone ash of 9.3% above that obtained for the group not receiving cod liver oil. For the two groups receiving cod liver oil, the average bone ash values for the ad libitum fed group were almost identical with those for the group on restricted feeding; i.e., 58.9 and 58.6%, respectively. In considering the data triad by triad, there were four cases in which the restricted rat had the higher bone ash, nine in which this rat had the lower amount, and three in which the values were practically identical. Although the preponderance of data might appear to indicate that the animals on the ad libitum regimen had the higher content of ash, statistical analysis showed that the odds were only 7 to 1 that the differences were significant.

It is obvious from this study that the additional amount of the basal ration as consumed by the ad libitum fed rats did not result in calcification beyond that obtained in their litter mates on restricted food. One may conclude, then, that when fairly generous amounts of calcium and phosphorus are fed daily apart from the ration a difference in the quantity of a calcium poor food eaten by the animals being compared is of minor consequence insofar as bone calcification is concerned. Under the conditions established in this regimen, therefore, a system of control feeding need not be adopted.

TABLE 3

Comparison of the average daily consumption of calcium and phosphorus in series 1 and 2

	SERIES 1		SERIES 2	
	Paired-feeding groups	Ad-libitum group	Paired-feeding groups	Ad-libitum group
	gm.	*gm.*	*gm.*	*gm.*
Basal ration, average daily intake	6.5	9.4	5.3	8.0
Calcium—in basal ration, average daily intake	0.033	0.048	0.001	0.001
Calcium—in adjuvants, daily intake	0.001	0.001	0.038	0.038
Calcium—total, average daily intake	0.034	0.049	0.039	0.039
Phosphorus—in basal ration, average daily intake	0.027	0.040	0.009	0.013
Phosphorus—in adjuvants, daily intake	0.007	0.007	0.028	0.028
Phosphorus—total, average daily intake	0.034	0.047	0.037	0.041
Calcium: phosphorus ratio	1: 1	1: 0.95	1: 0.96	1: 1.06

Comparison of the two technics. As seen in table 3, the average intake of calcium and phosphorus eaten by the animals in the two series was almost identical except for the ad libitum group in series 1. These latter animals consumed, as a daily average, 14 mg. calcium and 12 mg. phosphorus more than did their litter mates on paired feeding. This increased consumption of these bone calcifying elements must be considered responsible for the increase (3.4%) in bone ash of this group over that of their litter mates receiving cod liver oil and a restricted amount of food. For series 2, although the ad libitum group ate, as an average, 2.7 gm. more of the basal ration daily than did their litter mates on paired feeding, the

average intakes for both groups were practically identical in respect to calcium and nearly so for phosphorus (the inclusion of a low-ash casein in the ration made this possible). This similarity in the amount of these elements consumed by both groups is reflected in the lack of difference in bone ash values of the two rats of the triad receiving cod liver oil.

A consideration of the bone ash values for the rats of the two series indicates that the two rations were similar in respect to calcifying properties. For the animals receiving no vitamin D averages of 51.7 and 53.6% ash were obtained for series 1 and 2, respectively. For the pair mates receiving cod liver oil, the averages were practically identical for the two series; i.e., 58.0 and 58.6%. A striking resemblance also is seen between these values and that of 58.9% for the litter mates eating ad libitum in series 2.

The adaptability of the two technics to vitamin D assay work in which bone ash values are used as a criterion for calcification should be considered. For calcium-poor foods, which could be administered in a large quantity without affecting the ratio or total intake of calcium and phosphorus, the regimen as used in series 1 would be recommended because of ease of administration of both adjuvants and basal ration. If calcium rich foods were superimposed on this ration, the resulting change in level and ratio of salts might induce calcification which should not be attributed to vitamin D activity. With such foods ration 2 is advocated; since, by modifying the salt adjuvant, the desired ratio and level of calcium and phosphorus may be maintained, even though different quantities of the same food are being fed. This technic was used successfully in the determination of vitamin D potency of milk, a study which will be reported soon.

CONCLUSIONS

Data are presented which indicate that, in studies on bone calcification, it is desirable to equalize the mineral intake of the animals being compared. Two technics have been presented whereby such control is possible. One procedure

involves the paired-feeding method, the other provides for the feeding of the bone calcifying salts separate from the ration. The application of these technics to vitamin D assay studies in which bone ash values are used as a criterion is discussed.

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A COMPARATIVE STUDY OF THE GROWTH-PROMOTING AND BONE-CALCIFYING EFFECTS OF SEVERAL CARBOHYDRATES [1]

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ONE FIGURE

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The suggestion that lactose might be unique among other carbohydrates was mentioned by Mathews ('21) who noted its universality in the food provided by nature for all mammals during the period of greatest galactolipin synthesis. Since then numerous investigators have compared the physiological effect of diets containing milk sugar with those containing other naturally occurring carbohydrates. The problem has been approached from many angles. Irrespective of the criterion used, the action of lactose has been reported as exhibiting a lack of conformity with that of other carbohydrates, with the possible exception of galactose. One is led to conclude that these two sugars have a physiological role not participated in by other saccharides.

This paper deals with studies which were started in 1931. The data corroborate the previously reported stimulating action of lactose on the calcification of bones but do not support the contention that this carbohydrate has a specific effect on growth. A subsequent paper will deal with mineral metabolism as influenced by lactose and other carbohydrates.

[1] A portion of these data was presented before the annual meeting of the American Association of Dairy Science, June, 1933, Urbana, Illinois.

EXPERIMENTAL

The animals used in this experiment were healthy, 21-day-old albino rats which had been selected from the stock colony of the nutrition laboratories of the Home Economics Department. The ration of their progenitors, their age and weights at weaning, and the conditions under which they were caged during the experiment are discussed in detail in a previous publication by Outhouse, Smith and Merritt ('37). The assessment of the growth induced by the various rations was made on the basis not only of gains in body weight over the experimental period but also of body length. The latter value was determined at the end of the experiment by measuring the distance between the nose and anus of the etherized animals and represents final body length rather than gain in length. The criterion adopted for the study of the bone-calcifying action was the amount of ash deposited in the bones of the rats after 28 or 35 days on the rations. The data are expressed in terms of the percentage of ash in the fat-free, moisture-free bone. The methods used in preparing the bones for ashing are discussed in a publication by Outhouse, Macy and Brekke ('28).

The sugars fed were of C.P. grade.[2] The lactose was the type which naturally occurs in milk, and the galactose was the d-isomer. Neither gave a test for sterols; hence, they were not extracted with fat solvents. The lactose was free from ash; but the galactose, upon analysis, was found to contain 0.16% of calcium. The extra amount of this element consumed by the animals on the galactose supplement would approximate 1 mg. daily.

The quantity of lactose fed was determined on the basis of the following consideration. In earlier unpublished experiments 30 cc. of cow's milk daily was found to be the minimum quantity which, in the absence of cod liver oil, would insure normal calcification in rats. Therefore, an amount of lactose equivalent to that occurring in this quantity of milk

[2] Bakers-analysed lactose and sucrose were used and the galactose was a Pfanstiehl preparation.

was selected as the minimum for series 1, in which lactose was fed as a daily supplement. Animals receiving this level (i.e., 1.2 gm. daily) consumed, as an average, 23% of their calories in the form of lactose. For series 2 and 3, lactose was mixed with the ration at a level of 25%. In this case it contributed 26% of the total calories. The feeding of galactose was carried out at only one level; i.e., 0.6 gm. daily, a quantity which is equivalent to 95% of the galactose content of 1.2 gm. lactose.

Series 1. In this series, as in series 2, female rats were prepared for the experiment by depleting their bodies of whatever stores of vitamin D they may have had at the end of the nursing period. The ration used for this purpose consisted of a low-ash casein (Swanson, '30) 18, salt mixture no. IV (Osborne and Mendel, '18) 4, and starch 78%. Powdered spinach [3] and dried brewers' yeast [4] supplied all the necessary vitamins with the exception of vitamin D. After being fed this ration for 7 days, the animals were then selected for litter mate groups on the basis of weight and given the experimental ration for the following 28 days.

The experimental regimen employed consisted in feeding ad libitum a ration poor in calcium and phosphorus, together with daily supplements of a calcium- and phosphorus-rich mineral mixture and the necessary vitamin-carrying foods. The basal ration was composed of low-ash casein 18, starch 79.4, and salt mixture XXX 2.6%. The minerals necessary for the calcification of bones were supplied in the form of salt mixture no. IV at a level of either 287 mg. or 384 mg. daily. Vitamins A, B and G were supplied daily by 50 mg. of powdered spinach and 400 mg. of dried brewers' yeast. This ration was used in a previous study by Outhouse, Smith and Merritt ('37) and was found to support satisfactory calcification in the presence of vitamin D. Three litter mate rats were used in each group. All were fed the above ration and supplements.

[3] Powdered spinach was purchased from the California Vegetable Concentrates, Inc., Los Angeles, California.

[4] Dried brewers' yeast was obtained from the Northwestern Yeast Company, Chicago, Illinois.

One rat received no additions, one was given lactose or galactose, and the third rat was fed a 50 mg. dose of cod liver oil daily.[5] Lactose was fed at two different levels; i.e., 1.2 gm. and 2.0 gm. daily. In order to secure complete ingestion of the salt mixture and sugars, all adjuvants were mixed together with a small portion of the basal ration and placed in the cage early in the morning. When these were eaten, the basal ration was given ad libitum.

Series 2. In this study lactose was compared with starch, sucrose and cod liver oil. The carbohydrates and inorganic salts were mixed into the ration at a constant percentage. Four female rats, depleted of vitamin D and approximately identical in weight, were selected from the same litter. Four modifications of the same diet were prepared. The basal ration was complete in all necessary factors except vitamin D and had the following composition: low-ash casein 18, starch 67, salt mixture no. IV 4, yeast 10, and spinach 1%. When sucrose or lactose was fed, it comprised 25% of the diet and replaced an equivalent amount of starch. One rat was given the above ration; one the same ration plus 50 mg. cod liver oil daily; a third received the ration containing 25% lactose; and the fourth rat was fed the one containing 25% sucrose. These rations will be referred to as the starch ration, the cod liver oil ration, the lactose ration, and the sucrose ration, respectively. In order to avoid differences in intake of calories and mineral salts, a system of control feeding was adopted. All animals were allowed the same quantity of food; that one having the poorest appetite limited the amount that the others received. In order to equalize the caloric intake of all four animals of the group, the three which did not receive cod liver oil were given the same quantity of corn oil.[6] The experimental period lasted 28 days.

Series 3. In this study the technic differed from that in the preceding series in that male rats were used and the preliminary vitamin D-depletion period was not employed. The

[5] The E. L. Patch Company, Stoneham, Massachusetts, supplied the cod liver oil.
[6] Mazola oil was used.

animals, at weaning, were placed on the experimental rations and were maintained on them for 5 weeks. The age of the animals at the end of the period was 56 days the same as in series 1 and 2. Lactose and sucrose only were compared. The carbohydrates were mixed into rations which had the following composition: low-ash casein 18, starch 47, salt mixture no. IV 4, yeast 6, and lactose or sucrose 25%. Carotene,[7] administered daily in $\frac{1}{100}$ mg. doses, replaced spinach as the source of vitamin A. The paired-feeding technic was employed.

DATA AND DISCUSSION

The data accumulated in these three series are tabulated in tables 1 to 4 and will be discussed collectively. The rats apparently tolerated the doses of lactose and sucrose well as judged by the fact that there were no evidences of diarrhea or digestive disturbances at any time. However, the feces of the lactose-fed animals were bulkier than those excreted on the other rations. In practically all of the litter mate groups the animal eating the sucrose ration had the poorest appetite. In series 3 it was this rat which limited the quantity of food consumed by the pair. In series 2 the food alloted to the quartet was frequently determined by the sucrose-fed rat and just as often by the one on the starch ration.

INCREMENT IN SIZE

As influenced by lactose. The growth response of the rats to lactose was found to be dependent, to a considerable extent, on the type of feeding employed. Under conditions of ad libitum technic the rats fed this disaccharide made greater gains than did their litter mates on the basal ration in two of the three experiments (table 1). Under conditions of isocaloric feeding, however, the ingestion of lactose did not induce greater growth, as assessed by weight gains or by final body length, than was found in litter mates receiving

[7] Crystalline carotene, purchased from the S. M. A. Corp., Cleveland, was dissolved in Wesson oil.

TABLE 1

Average values for food intake, gain in weight, and bone ash of rats in series 1

EXPERIMENT NO.	CARBOHYDRATE		SALT MIXTURE NO. IV DAILY	NUMBER OF GROUPS	GROUPS FED BASAL RATION			GROUPS FED BASAL RATION AND COD LIVER OIL			GROUPS FED BASAL RATION AND CARBOHYDRATE		
	Kind	Amount daily			Food intake (28 days)	Gain in weight (28 days)	Bone ash[1]	Food intake (28 days)	Gain in weight (28 days)	Bone ash	Food intake (28 days)	Gain in weight (28 days)	Bone ash
		gm.	*mg.*		*gm.*	*gm.*	%	*gm.*	*gm.*	%	*gm.*	*gm.*	%
1	Lactose	1.2	287	8	153	33	50.1 ± 1.9[2]	234	69	57.9 ± 1.5	144	41	54.7 ± 2.2
2	Lactose	1.2	384	9	166	33	55.2 ± 2.2	218	62	60.9 ± 1.4	146	34	58.4 ± 2.0
3	Lactose	2.0	384	9	167	31	56.0 ± 2.1	211	55	61.7 ± 1.2	138	41	60.0 ± 1.0
4	Galactose	0.6	287	11	157	33	55.6 ± 1.9	226	71	60.3 ± 1.8	153	35	56.5 ± 1.6

[1] Calculated on basis of dried, alcohol-ether extracted bone.

[2] Standard deviation.

non-lactose rations. This was true for series 2 in which the lactose ration was compared with the starch, the sucrose, and the cod liver oil rations and in series 3 in which lactose was compared with sucrose. Statistically significant differences (Student, '15–'17) were not found on considering the data from the standpoint of averages for the groups or of differences between the pairs. The slight superiority in weight gains by the lactose-fed rats over those of litter mates on the basal ration in experiments 1 and 3 in series 1, as mentioned above, can therefore be explained on the basis of a greater calorie intake.

As influenced by sucrose. This disaccharide, when compared with starch or lactose, was found to have no effect whatsoever on weight gains or final body length of female rats in series 2 or on weight gains of the male rats in series 3.

As influenced by galactose. The growth promoting properties of galactose were observed only under conditions of ad libitum feeding and at a single level; i.e., 0.6 gm. daily. As seen in experiment 1 (table 1), this monosaccharide had no influence on either appetite or gain in weight in addition to that observed in litter mate animals receiving the basal food.

As influenced by cod liver oil. In both series in which cod liver oil was fed, the greatest gains were made by the animals receiving this supplement. In series 1, however, the strikingly accelerated growth was accompanied by a greater food intake and should be considered as being due primarily to this. Conclusive proof that some of this gain may have been the result of the ingestion of cod liver oil, is given in series 2. All members of the quartet received the same quantity of food, yet the animal on the cod liver oil ration gained more than the one on the starch ration in sixteen of the twenty groups. A statistical analysis of these data yielded chances of 9999 to 1 that the differences were not accidentical. Odds of a similar magnitude were obtained for differences in final body length of these animals.

These data show the growth promoting value of cod liver oil when added to these rations. In addition, they are of

primary significance in that they demonstrate that, if rations are not equal in their growth stimulating qualities, the method of control feeding can be relied upon to indicate that difference.

Comparison of data with those of other investigators. The results obtained in this study are not greatly variant from those reported in the literature. In most of the investigations in which ad libitum feeding technic was used the various authors have interpreted their data as indicating a difference between the growth promoting properties of lactose and other carbohydrates fed. The inclusion of milk sugar in a variety of rations was felt by several to be especially productive of growth (Kline, Keenan, Elvehjem and Hart, '32; Whittier, Cary and Ellis, '35). On the other hand, Jarvis ('30) and Koehler and Allen ('34) found lactose inferior to sucrose as a growth stimulator. Similar results were obtained by Whittier, Cary and Ellis ('35) when adult animals were used. Mitchell ('27) reported that rations containing more than 30% of lactose induced less growth than did those containing comparable amounts of starch, dextrin, maltose, or sucrose. In later work Mitchell and Dodge ('35) found that gains were subnormal on lactose containing diets only when this disaccharide comprised 70% of the ration. In none of these studies have food intakes been reported. With the exception of Whittier, Cary and Ellis ('35) no one has suggested that the differences in weight gains may have been due to a possible disparity in the amount of food consumed by the animals being compared. When these authors fed lactose under conditions of control feeding there was found only slight superiority of lactose over sucrose. If an incongruity exists between their results and those reported in this paper, it may be due to differences in the quantities of lactose fed. Ariyama and Takahasi ('29), feeding rats 30% carbohydrate in iso-caloric rations, observed greater growth with lactose than with sucrose and less than with starch. The slight differences observed and the small number of animals used should make any conclusions of dubious value. Certainly the data herein

reported for weight gains in the fifty-four pairs of male rats and the twenty quadruple groups of female rats and for body lengths in fifteen of the latter twenty groups fail to show any superiority for either of these sugars, when fed in rations in which they were substituted for starch at a level of 25% of the diet. One may conclude, therefore, that when fed at moderate levels lactose is equivalent to sucrose in growth-promoting value and that both are comparable to starch.

Conclusions. In the studies in which a system of control feeding was employed it was found that lactose had no greater power than sucrose to stimulate increase in weight or length, and that neither had exhibited, at the levels fed, any growth promoting value in addition to that given by the polysaccharide starch. With these findings in mind, one would hesitate to interpret the results of the ad libitum series as indicating a special growth promoting property of lactose. Although in series 1 galactose was not observed to stimulate gains in weight, a statement as to its growth promoting value should be withheld until it has been fed under conditions of control feeding.

ASH CONTENT OF BONES

As influenced by sucrose. A comparison of sucrose with starch was made in series 2 (table 2). Of the twenty-one pairs of rats there were five in which both members had approximately the same amount of bone ash, eight which favored the starch ration, and eight which favored the sucrose ration. No differences could be found on statistical analysis of these data (odds of 2 to 1) or in the averages for the two groups. Therefore, the substitution of 25% sucrose for an equivalent portion of starch had conferred no additional calcifying properties to the ration.

As influenced by lactose. In the two series in which lactose was compared with starch, the disaccharide was found to have the greater calcifying power. For series 1 individual litter response to the three diets are given in figure 1. Litter variation is seen to be great. Yet, in all but two cases, the animal

TABLE 2

Gains in weight and final-body lengths for the twenty groups of animals in series 2

LITTER MATE GROUPS	FOOD INTAKE (28 DAYS)	GAIN IN WEIGHT (28 DAYS)				FINAL BODY LENGTH			
		Starch ration	Cod liver oil ration	Lactose ration	Sucrose ration	Starch ration	Cod liver oil ration	Lactose ration	Sucrose ration
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*mm.*	*mm.*	*mm.*	*mm.*
3956–59	160	31	41	38	32	152	152	150	152
3970–73	158	38	50	37	43	156	163	160	156
3981–87	192	37[1]	46	43[1]	36[1]	158	162	155	155[1]
3988–91	115	12	24	13	12	....	....	....	....
4001–05	142	28[1]	34	28	29	154	153	148	145
4006–12	152	28[1]	52	39[1]	37[1]	....	....	....	....
4016–21	152	34	42	32[1]	27[1]	154	157	151[1]	149[1]
4029–34	151	32	41	36[1]	38[1]	146	155	156	156
4045–48	179	36	49	43	43	....	....	....	....
4203–09	198	51	54	43	47	158	162	158	162
4205–10	198	53	56	43	53	160	158	153	159
4278–85	117	25	26[1]	24[1]	26[1]	....	....	....	....
4411–14	181	40	42	35	43	160	159	157	157
4415–18	155	26	30	24	29	152	156	150	157
4448–53	163	36	40	34	44	....	....	....	....
4517–20	121	18	14	9	12	140	145	141	143
4726–29	193	42	48	44	46	166	166	165	160
4730–33	148	28	29	29	29	153	155	150	152
4734–37	186	45	42	32	38	157	160	156	156
4738–41	137	23	25	29	30	153	159	153	151
Average	160	33	39	33	35	155	158	154	154
Standard deviation	25.6	10.0	11.2	9.5	10.6	5.9	5.0	5.5	5.2
Probable error	17.3	6.7	7.6	6.4	7.1	4.0	3.4	3.7	3.5

[1] Average for two animals.

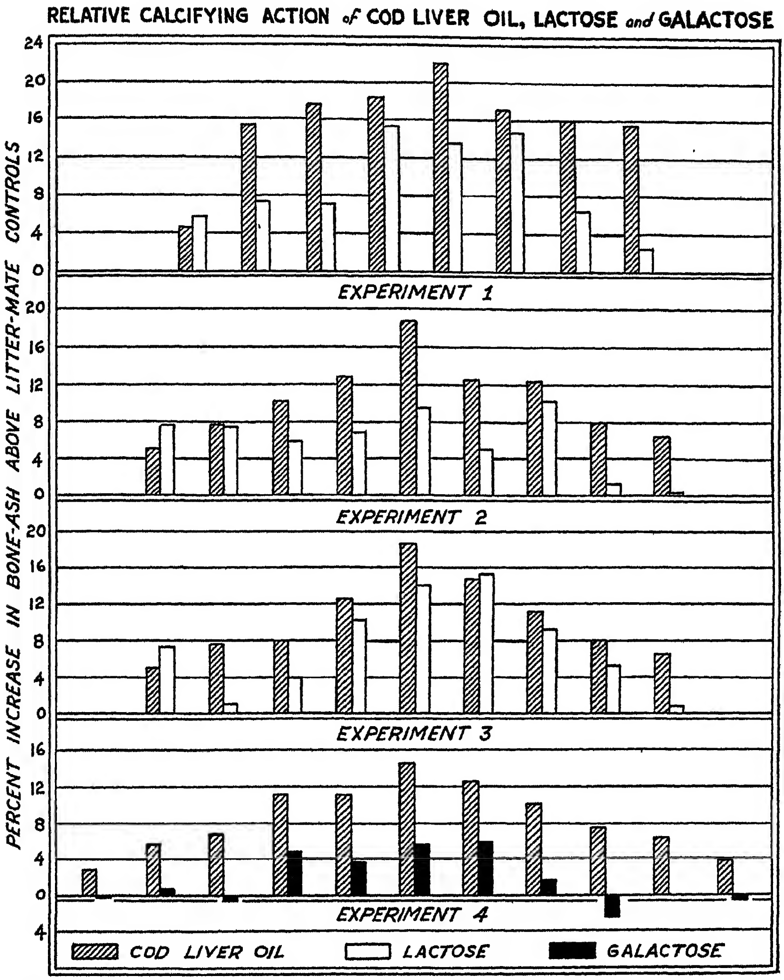

Fig. 1 The calcifying action of lactose, galactose and cod liver oil in relation to the basal ration is graphically presented here for each litter mate group in series 1. In plotting the data the relative amount of ash in the bones of the rat on the basal food was placed at 0, forming a base line. The increase or decrease from this value, observed for the other two rats of the litter mate groups, is represented in columns above or below this line in terms of per cent.

receiving lactose had significantly more ash in its bones than had its litter mate on the basal ration. For these three experiments the statistical odds ranged from 999 to 1 to 2499 to 1 that the results were not fortuitous. In the control feeding studies in twenty of the twenty-one groups, the lactose-fed rats had the greater bone ash. Odds greater than 9999 to 1 indicate practical certainty that lactose is superior to starch as a bone-calcifying agent.

The data presented in the two preceding paragraphs would indicate that lactose possesses calcifying properties not exhibited by sucrose. Proof of this is obtained in the studies in which a direct comparison was made between these disaccharides. A greater bone ash in the lactose-fed animal was found in twenty of the twenty-one pairs in series 2 (table 4) and in fifty-four of the fifty-six pairs in series 3 (table 3). For both series the statistical odds were greater than 9999 to 1 that the results were not accidental. It is of interest to note that the average values for the males in series 3 and for the females on comparable rations in series 2 were practically identical.

As influenced by galactose. As seen in figure 1, the responses of the rats to this monosaccharide were inconsistent. A statistical analysis of the data failed to show significant differences between these animals and those receiving the basal ration only. It is possible that, if the galactose had been fed in such a manner as to approximate the rate at which galactose is made available for absorption following the enzymic splitting of lactose, different results might have been obtained. Probably the only conclusion that can safely be drawn from this study is that, under the conditions employed, the feeding of d-galactose was not attended uniformly by increased deposition of ash into the skeleton, whereas comparable amounts of galactose fed in the form of lactose induced more normal calcification.

As influenced by cod liver oil. In general the highest values for bone-ash found in this study were in the rats receiving cod liver oil (see fig. 1 and tables 1 and 4). In six of the

TABLE 3

Individual responses in appetite, growth and calcification of fifty-six pairs of males studied in series 3

LITTER MATE PAIR NO.	FOOD INTAKE (35 DAYS)	GAIN IN WEIGHT (35 DAYS)		ASH CONTENT OF BONES[1]	
		Lactose ration	Sucrose ration	Lactose ration	Sucrose ration
	gm.	*gm.*	*gm.*	%	%
1	198	55	65	57.7	51.1
2	225	60	62	56.5	52.7
3	241	87	73	56.8	54.8
4	192	58	66	54.6	51.5
5	221	74	75	55.3	51.5
6	218	60	60	54.7	54.3
7	194	38	46	57.1	52.9
8	215	60	62	57.0	55.8
9	196	50	59	55.8	52.9
10	227	63	65	56.0	55.0
11	248	77	72	56.7	55.1
12	225	63	76	57.9	56.9
13	226	76	60	57.5	54.2
14	164	37	43	56.1	54.4
15	251	60	71	54.8	50.7
16	236	62	69	57.1	53.1
17	261	82	84	57.7	52.5
18	238	71	71	58.9	53.9
19	238	74	74	55.6	53.6
20	234	77	75	55.5	53.6
21	231	74	73	56.1	52.7
22	310	90	88	58.6	55.0
23	235	68	71	56.5	51.9
24	264	83	81	57.9	53.6
25	214	41	47	56.8	52.4
26	261	81	84	57.6	54.4
27	240	63	84	56.2	54.4
28	296	92	91	58.7	56.3
29	244	79	76	57.8	52.0
30	306	91	104	59.5	54.8
31	373	121	106	59.1	56.6
32	221	74	74	57.1	52.0
33	215	78	73	58.1	57.5
34	241	80	80	58.4	58.3
35	209	64	70	55.1	54.6
36	246	78	77	56.3	53.8
37	262	84	79	58.3	52.6
38	201	68	63	56.2	52.7
39	214	74	72	54.7	52.6
40	208	80	63	56.4	53.7
41	188	59	62	56.8	53.1
42	200	68	72	57.1	53.3
43	185	60	66	55.3	50.3
44	165	49	55	52.2	51.0
45	175	69	54	54.4	48.5
46	161	47	49	54.3	45.5
47	134	40	42	52.8	48.6
48	195	56	57	55.5	55.0
49	170	61	60	54.0	51.2
50	166	58	56	55.2	55.7
51	150	52	55	55.7	51.8
52	143	46	45	54.5	52.7
53	149	43	48	52.8	48.8
54	157	50	52	56.3	53.9
55	153	....	....	57.9	51.2
56	144	....	....	53.5	48.9
Average	216	67	68	56.3	53.1
Standard deviation	45.8	16.0	13.9	1.6	2.3
Probable error	30.9	10.8	9.4	1.1	1.6

[1] Calculated on basis of dried, alcohol-ether extracted bone.

twenty-one cases in the control feeding series the lactose-fed rat had a higher bone-ash content than those receiving the above supplement, yet the statistical odds were 140 to 1 that cod liver oil was the better calcifying agent.

TABLE 4

Ash content of bones of rats in series 2

LITTER MATE GROUPS	FOOD INTAKE (28 DAYS)	ASH CONTENT OF BONES[1]			
		Starch ration	Cod liver oil ration	Lactose ration	Sucrose ration
	gm.	%	%	%	%
3956–59	160	53.8	58.6	58.4	56.6
3970–73	158	55.9	58.5	57.9	56.4
3981–87	192	55.8[2]	58.5	59.7[2]	55.4[2]
3988–91	115	54.0	57.6	54.5	51.3
4001–05	142	51.8[2]	55.8	55.6	52.6
4006–12	152	53.1[2]	58.0	55.8[2]	54.7[2]
4016–21	152	55.8	57.2	56.5[2]	52.2[2]
4029–34	151	50.1	55.5	55.7[2]	54.5[2]
4045–43	179	53.1	58.0	56.3	52.2
4203–09[3]	198	56.4	57.9	56.6	56.6
4205–10[3]	198	55.4	56.8	57.4	53.9
4278–85[3]	118	49.7	55.6	53.6	49.6
4279–81[3]	117	50.7	54.4	53.6	50.6
4411–14	181	55.7	58.4	55.7	52.8
4415–18	155	53.0	57.6	58.5	54.0
4448–53	165	53.2	57.0	58.4	52.4
4517–20	121	51.2	53.7	53.7	49.4
4726–29	193	56.1	58.0	58.2	53.2
4730–33	148	53.6	58.9	58.1	53.5
4734–37	186	53.6	60.0	55.9	55.1
4738–41	137	55.6	58.7	57.1	55.9
Average	153	53.7	57.4	56.5	53.5
Standard deviation	26.6	2.0	1.5	1.7	2.1
Probable error	17.9	1.3	1.0	1.1	1.4

[1] Calculated on basis of dried, alcohol-ether extracted bone.

[2] Average for two animals.

[3] Litter mate groups.

The addition of vitamin D in series 2 did not produce bones of maximal ash content (except in two cases). The values, however, are consistent with data reported earlier for rats receiving restricted food and cod liver oil (Outhouse, Smith and Merritt, '37).

Comparison of these data with previously published work. This calcifying activity of milk sugar has been noted previously. Thus, Jarvis ('30) had reported lactose to be superior to sucrose for rats; and Kline, Keenan, Elvehjem and Hart ('32) had found that, in contrast to maltose or citric acid, it induced chicks on rachitogenic ration to deposit a greater amount of ash in their skeletons. Whittier, Cary and Ellis ('35) have presented data secured under conditions of control feeding. In three of four pairs of rats, fed approximately 4 months, the lactose-fed rat had a greater body-ash content than the sucrose-fed rat. In three pairs of pigs, the ones receiving lactose showed a higher ash content in the edible portion of their carcasses than was found in the sucrose-fed pair mates. This is an indication that the soft tissue as well as the skeleton may be influenced by the inclusion of moderate amounts of lactose in the diet. Possible proof of this is found in the work of Mitchell and Dodge ('35). In their studies on high levels of lactose ash was deposited in the eye to the extent that the calcium content was approximately twice as great as that found in control animals. Evidences of cataractous eyes were not found in the animals during the short period of these studies herein reported.

Conclusions. The results obtained in these studies demonstrate that, when lactose comprised 23 to 26% of the caloric intake of rats, better calcified bones resulted than when comparable amounts of sucrose were given or when starch was the only carbohydrate fed. Galactose in amounts equivalent to that found in these levels of lactose caused no increase in bone ash. Inasmuch as the mineral intake was controlled in each of the two technics employed, it is felt that the observed variations in bone ash induced by these four carbohydrates must be due to differences in their physiological values.

SUMMARY

Under conditions of controlled mineral intake lactose, sucrose, and galactose were fed to young litter mate rats in amounts equivalent to 23 to 26% of their caloric intake. With ad libitum feeding the supplementation of the basal diet with lactose induced greater gains in rats than were obtained in litter mates on the basal diet, but only when the former consumed appreciably more calories. On paired feeding no alteration in growth was observed when 25% of sucrose or lactose replaced an equivalent amount of starch in the basal ration. Of these carbohydrates lactose was the only one which caused an acceleration in bone calcification. This effect was found under both ad libitum and control feeding conditions. In comparison with cod liver oil, however, this sugar, at the levels fed, did not induce as well calcified bones.

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THE NUTRITIVE VALUE OF THE PROTEINS OF NUTS IN COMPARISON WITH THE NUTRITIVE VALUE OF BEEF PROTEINS

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It is a singular fact that in human nutrition diets are universally planned on the basis of the chemical composition of available foods, while in animal nutrition the balancing of rations is commonly undertaken on the basis of the contents of digestible nutrients in animal feeds. The observation that human foods are ordinarily more digestible than animal feeds is only a partial justification for this difference since many vegetables and fruits are relatively indigestible. The digestibility of human foods has not been systematically studied, although many scattered determinations may be found in the literature. The practical value of such work is obvious.

Nutrients undergo wastage in the body not only in the gastrointestinal tract, but also in metabolism. The metabolic wastage of protein is conditioned to a large extent by its content of those amino acids indispensable for animal life, and to some extent by the protein content of the diet fed. A metabolic wastage of all organic nutrients is represented in the specific dynamic effect of a meal. Clearly, the complete evaluation of a food or a diet as a source of nutrients to the animal must consider all manner of wastage in the course of its assimilation, since any food is of value to the body, not in proportion to what the food contains, but in proportion to what the body can extract from it for its own uses. This conception of the

'net value' of a food, as contrasted with its 'gross value' measured by a chemical analysis, is the contribution of H. P. Armsby, the significance of which seems to be appreciated only in animal nutrition.

The term 'biological value' of a protein was first introduced by Thomas to designate that percentage of the absorbed protein that is retained in the body for its own uses. Hence the magnitude of this value will vary inversely with the wastage of protein in metabolism. The digestion coefficient and the biological value of the protein fraction of a food would seem, therefore, to define completely its nutritive value, and, in combination with the percentage of protein in the food, to define what has been called the 'net protein content' of the food (Mitchell, '27).

Quantitative studies of the nutritive value of foods are among the most practical contributions of the science of nutrition. In particular, they facilitate the nutritional classification of foods, and a better understanding of their nutritional equivalence. For example, on the basis mainly of their high contents of protein and of fat, meat and the oil-bearing nuts have been considered equivalent in nutrition; in other words, nuts have been proposed as adequate meat substitutes. However, this nutritive equivalence may be more apparent than real, since it is based almost entirely upon chemical composition.

It will be the purpose of this study to assess quantitatively the protein value in nutrition of nuts as compared with meat (beef); in this paper, results obtained on the almond, the Brazil nut, the filbert, the cashew nut, and the English walnut will be reported. These five nuts with the peanut and the pecan previously studied (Mitchell, Burroughs and Beadles, '36) are among the most popular varieties consumed in this country.

The digestibility of the protein of nuts has not been very thoroughly or extensively studied. Holmes ('18) has reported that the protein of peanut flour is digested by human subjects to the extent of 86%, while Cajori ('18), in experiments on

both men and dogs, obtained coefficients of digestibility of 80 to 92% for the protein of diets containing unreported amounts of nuts. On the other hand, Jaffa ('03) has observed among the fruitarians of California a much lower digestibility of the protein of rations containing large amounts of nuts; for rations containing protein derived largely from walnuts (63 to 86%) eight coefficients averaging 74.4 were obtained; in two cases, rations containing large proportions of peanut protein were digested to the extent of 87 and 78%, but in five digestion trials with rations containing pecans, furnishing generally about half of the total protein intake, an average coefficient of only 70 was obtained. Jones and Waterman ('22) have reported a low digestibility of arachin, the main protein of the peanut, in enzyme digestion experiments. A variable digestibility of nut protein is to be expected according to Heupke ('31) depending upon the completeness of mastication; an extremely low digestibility may follow poor mastication. We have obtained (Mitchell, Burroughs and Beadles, '36) coefficients of true digestibility with rats of 71 for pecan proteins and 96 for peanut proteins.

A number of feeding experiments to test the nutritive value of nut proteins, either alone or in combination with other food proteins, have been published. Sure ('20) was unable to secure good growth in rats on rations containing as high as 18% of arachin or of the mixture of arachin and conarachin extracted from peanut meal by salt solutions. Using the numerical method of Osborne, Mendel and Ferry to express the nutritive value of dietary protein (gain per gram of protein consumed), Maynard, Fronda and Chen ('23) obtained higher values for peanut oil meal (average of 1.45) than for corn meal (average of 1.18) at a protein level of 9%, together with some indication of a moderate supplementary relation between the proteins of these two foods. Shiba and Koyama ('23) also obtained indications of a high nutritive value of peanut protein fed at a 14% level, while Pian ('30) in nitrogen balance studies obtained a biological value of only 59 for the proteins of the Chinese peanut with a diet containing 10% of

protein. In the previous study (Mitchell, Burroughs and Beadles, '36) from this laboratory on the nutritive value of nuts, biological values of 58 for the peanut and 60 for the pecan were obtained with rations containing 7.6% of digestible protein calories, using rats as subjects. These investigations are somewhat difficult to reconcile.

The supplementary relations between the protein fraction of any high protein food and of wheat flour is of great practical significance in dietetics, because wheat endosperm proteins themselves possess such a low biological value. Johns and Finks ('20) found that a bread containing 25 parts of peanut flour and 75 parts of wheat flour furnished adequate proteins and water-soluble vitamins for 'normal' growth when fed at a 10% protein level. Eddy and Eckman ('23) have confirmed the high nutritive value of a mixture of peanut and wheat flour and have compared this flour with a mixture of wheat flour and meat residue, also containing 6 to 7 parts of wheat flour protein to 10 parts of total protein. The peanut flour mixture promoted somewhat better growth and markedly better reproduction than the meat residue mixture at the same level of protein intake. This superiority of the peanut flour mixture is attributed by the authors of the report to a superior supplementing value of the peanut proteins, although they admit that the two flour mixtures were the sole source of vitamin B (the complex) as well as of protein. The meat residue was prepared in such a way that the major part of the contained water-soluble vitamins would be removed.

In unpublished experiments we have shown that a mixture of peanut flour and patent white flour in the proportion of 1 to 3, the nitrogen of which was derived from the two flours to the extent of 62 and 38%, respectively, was somewhat higher in biological value for young albino rats than either flour alone, but was much lower in biological value than a similar mixture of beef and white flour proteins, contrary to the above cited conclusion. The respective biological values, determined with ten rats in each test, were 59.6 and 80.5. The expected values if no supplementary relations existed are

54.3 and 67.9, respectively. The marked superiority of the proteins of the beef-white flour mixture over those of the peanut-white flour mixture was confirmed by a paired-feeding test (Mitchell and Beadles, '30).

Concerning the English walnut and the pecan, little work has been reported with reference to their values as sources of dietary protein. Cajori has obtained good growth in rats with walnut proteins ('20), and pecan proteins ('21) when fed at a concentration of 18%, but such qualitative data indicate merely that the proteins are complete in their amino acid constitution and are adequate for growth. The nutritive value of walnut proteins for rats and mice was more extensively studied by Mignon ('23), who obtained 'normal' growth with the total proteins of the walnut at a 12% level of protein, and about the same results with walnut globulin. The residual proteins after extraction of the globulins induced only an unsatisfactory growth even at an 18% level of intake. The gain per gram of protein consumed was not particularly high in any of these tests, never exceeding 1.39 gm. for rats or 0.50 gm. for mice.

In the investigations of nut proteins reported below it was expected that the most significant determinations of nutritive value would be obtained only by the use of quantitative methods involving the separation of the wastages of nitrogen in digestion and in metabolism. The biological values of the various protein mixtures were determined according to the most improved method developed in this laboratory (Mitchell, Burroughs and Beadles, '36).

PLAN OF EXPERIMENTS

The nutritive values of the proteins of the almond, English walnut, Brazil nut, cashew nut and filbert were studied by determining the nitrogen intake and outgo of the rats under conditions standardized so that significant biological values could be estimated (Mitchell, '24; Mitchell and Carman, '26; Mitchell, Burroughs and Beadles, '36). The rations used throughout were adequate for good growth in all known respects except for the protein mixture under study. They

contained 22% of fat, instead of the usual 10 to 12%, in the hope that the consistency would be such as to minimize scattering. Since this hope was not realized, all food portions after being weighed into the food cups were mixed with water to a thin consistency, an expedient which had the desired effect. In previous work, a level of 8% of dietary protein has been adopted, but with the higher fat content of the diets, the protein content was raised from 8 to approximately 9.2% in order to maintain constant the percentage of protein calories in the diet at 7.6%.

The biological values of the dietary proteins were determined upon groups of four to twelve growing rats, the initial weights of which varied from 50 to 120 gm. The plan of the experiments varied, from the simplest type, such as one in which a filbert ration was fed in period 1, an almond ration in period 3, and the standardizing ration containing 4% of egg protein in the intermediate period, to one extending over five or six periods in which three nuts were compared, with intervening standardizing periods. The Brazil nut, the filbert, and the cashew nut were compared directly with beef by feeding half of the group a nut ration and the other half a beef ration; then, after a standardizing period, the feeding was reversed. In such experiments as the latter, paired-feeding was used in order to obviate any possibility of error due to a variable intake of food.

The low-nitrogen ration used, containing 4% of whole egg protein, was the standardizing ration, upon which were determined the metabolic nitrogen of the feces per gram of dry matter consumed and the endogenous nitrogen per 100 gm. of body weight. These values were applied without adjustment to the preceding and following periods in those tests covering only three periods. The collection periods were of 7 days duration, with transition periods of at least 4 days.

The nuts to be tested were analyzed in the routine way, with the results given in table 1. The results previously reported for the peanut and the pecan (Mitchell, Burroughs and Beadles, '36) are included for comparison. The gross energy

value was determined with the bomb calorimeter. Before incorporation into the experimental rations, the nuts were finely ground, dried before a blower at temperatures not exceeding 50°C., extracted repeatedly (but not exhaustively) with ether, dried and again analyzed for nitrogen and fat. The beef round, containing the protein with which the nut proteins were compared, was trimmed of visible fat, dried before the blower, ground finely, extracted with ether, dried again and also analyzed for nitrogen and fat.

TABLE 1

The chemical composition of the nuts tested

DESCRIPTION	MOISTURE	CRUDE PROTEIN	ETHER EXTRACT	CRUDE FIBER	NITROGEN-FREE EXTRACT	ASH	CALCIUM	PHOSPHORUS	GROSS ENERGY IN CALORIES PER GRAM
	%	%	%	%	%	%	%	%	
Brazil nuts, fresh	3.50	16.25	69.75	4.48	2.85	3.17	0.195	0.664	7.55
Cashew nuts, fresh	4.39	19.52	48.70	1.20	23.72	2.47	0.041	0.507	6.48
Almonds, blanched	4.56	21.94	56.75	3.34	10.29	3.12	0.251	0.527	6.76
Filberts, fresh	3.70	13.50	70.14	5.26	5.55	1.85	0.100	0.305	7.57
Pecans, fresh	4.30	11.28	72.96	4.90	5.02	1.54	0.073	0.315	7.69
English walnuts, fresh	4.39	21.16	61.91	2.35	8.07	2.12	0.096	0.463	7.32
Peanuts, raw, without skins	5.24	28.25	51.26	1.89	10.84	2.52	0.049	0.553	6.85

The analyses of pecan nuts are the averages for three samples obtained from as many consignments. For Brazil nuts, two consignments are represented. The analyses of other nuts relate to one sample from one consignment.

Of the experimental rations, it may be said of all that they contained approximately 9.2% of protein, 22% of fat, 5% of the Wesson modified Osborne and Mendel salt mixture (Wesson, '32), 1% of NaCl, and 10% of sucrose. The fat was provided by the test sample, 8% of butter fat, 2% of cod liver oil, and lard in an amount needed to make up 22% of total fat. Vitamins B and G were provided by 1% of dried yeast and Harris vitamin powder given in separate small dishes at the rate of 5 mg. per gram of food; in most tests, however, these vitamins were supplied by giving each rat daily 3 to 5 drops of a solution containing for each 100 cc.

20 gm. of Harris vitamin powder and 20 gm. of sucrose. At times the dosage of yeast vitamin was increased in attempts (not always successful) to restore a failing appetite. All rations were completed by variable additions of corn starch. The gross energy content approximated 4.8 cal. per gram.

THE RESULTS OBTAINED

It seems unnecessary to give the nitrogen metabolism data and all of the calculations leading to the coefficients of true digestibility and the biological values obtained in these numerous experiments, since the method of calculation has been explained in previous publications from this laboratory. In all experimental periods the rats gained in weight, albeit the gain was quite variable. In comparative tests the attempt was made to equalize the intake of food, and in most cases this ideal was realized. The average coefficients of true digestibility and the average biological values, with their probable errors computed according to the classical method, are summarized in table 2. Again the results for the peanut and the pecan, previously reported (Mitchell, Burroughs and Beadles, '36), are included for comparison.

In digestibility and in biological value, the proteins of beef round are superior to those of any of the nuts studied; generally the difference in biological value is great, representing an improvement over the nut proteins of 25 to 50%. The difference in digestibility is not great, when beef proteins are compared with the proteins of the peanut, the Brazil nut, the cashew nut or the almond. However, the proteins of the pecan are relatively indigestible, 71%, while those of the English walnut are only 84% digestible. These facts should be considered in making any comparison of the nutritive value of meats and nuts, for example, the statement sometimes made that nuts are 'meat substitutes.'

While the biological values of all other nuts under the conditions of these experiments fall within the range 50 to 60, the cashew nut is a prominent exception, possessing a value of 72, only slightly below that of beef round, 76.

The filbert rations afforded us the first prominent instance of the circumstance noted occasionally (Kon, '28; Turk, Morrison and Maynard, '35) by others, namely, that biological values determined immediately after a period of low-nitrogen feeding tend to be higher than those obtained after periods of test-protein feeding. This has been interpreted (Chick, Hutchinson and Jackson, '35) as the result of a 'nitrogen debt' incurred during specific protein starvation. However, another possibility more in harmony with the very occasional appearance of this phenomenon (Mitchell, '28) is that in the preceding low-nitrogen feeding period the endogenous level of nitrogen excretion had not been attained, so that the biological values of the following period more than those of subsequent periods, are fictitiously high.

TABLE 2

The true digestibility and the biological value of nut proteins and of beef proteins

SOURCE OF PROTEIN	NUMBER OF TESTS	TRUE DIGESTIBILITY		BIOLOGICAL VALUE	
		Average and probable error [1]	Standard deviation [2]	Average and probable error [1]	Standard deviation [2]
Beef round	41	100	...	75.78 ± 0.41	3.86
Brazil nut	14	95.71 ± 0.22	1.24	54.07 ± 0.45	2.52
Cashew nut	22	96.23 ± 0.16	1.11	72.50 ± 0.66	4.56
Almond	19	93.95 ± 0.23	1.50	50.84 ± 0.37	2.37
Filbert	14	91.29 ± 0.20	1.09	50.29 ± 0.67	3.75
Pecan	10	70.71 ± 0.45	2.13	59.80 ± 0.50	2.32
English walnut	9	84.11 ± 0.22	1.00	55.89 ± 0.92	4.11
Peanut, raw	10	97.39 ± 0.27	1.27	57.9 ± 1.1	5.30
Peanut, roasted	10	96.15 ± 0.22	1.05	55.80 ± 0.77	3.59

[1] These probable errors do not measure reliably the significance of differences between averages for two different sources of protein when both have been tested with the same group of rats. In such cases, when the two results obtained with the same rat are considered as paired observations, and 'Student's' method of analysis applied, the difference between averages assumes a greater significance. Thus, judged by the probable errors in the above table, the difference in biological value between the proteins of raw and roasted peanuts is not significant, but when the average difference is considered as the average of a series of paired differences using 'Student's' method of analysis, it becomes highly significant (Mitchell, Burroughs and Beadles, '36).

[2] Standard deviations computed by the formula $s = \sqrt{\frac{\Sigma\delta^2}{n}}$

With the filbert, fourteen determinations of biological value made before a standardizing period averaged 50, while eleven determinations carried out immediately following a standardizing period averaged 63.

In determining which of these two values is the true one, the filbert was compared with the cashew nut (for which, all biological values were consistent) in a modified paired-feeding test in which paired rats are given the same amount of food and the level of the better protein under comparison is lowered until equal gains are secured. This method is explained in a previous publication from this laboratory (Mitchell, Burroughs and Beadles, '36). In eleven pairs of rats carried along in this way for 9 weeks (except for one pair discontinued at the end of 38 days), on the average, 1 gm. of digestible nitrogen from the cashew nut promoted the same rate of growth as 1.41 gm. of digestible nitrogen from the filbert. Analysis of the rats at the termination of the feeding period revealed no significant difference in the storage of nitrogen between pair mates.

Taking the biological value of cashew nut protein as 72, that of the filbert may be computed from the proportion $72:x::1.41:1$. The value of $x = 51$, approximately the same as the average of 50 obtained by the nitrogen metabolism method before a standardizing period. Therefore, this value is taken in preference to the higher value of 63, obtained after low-nitrogen feeding.

Referring to table 2 again, it will be noted that the standard deviations of the individually determined biological values range from 2.32 to 5.30, averaging 3.60. This is practically the same as the average precision previously noted (Mitchell, Burroughs and Beadles, '36) in this determination as carried out on growing rats by the method perfected in this laboratory, measured by an average standard deviation of 3.7.

SUMMARY AND CONCLUSIONS

The true digestibility and the biological value of the proteins of beef round and of five nuts, Brazil, cashew, almond, filbert and English walnut, have been determined in from nine to forty-one tests in each case, using growing albino rats as subjects.

Beef protein is superior to all of the nut proteins studied in both particulars, although the cashew nut is not greatly inferior, with a digestibility of 96 and a biological value of 72. All other nuts exhibit biological values under the conditions of these experiments ranging from 50 to 60.

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NUTRITIONAL WELL-BEING AND LENGTH OF LIFE AS INFLUENCED BY DIFFERENT ENRICHMENTS OF AN ALREADY ADEQUATE DIET

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ONE FIGURE

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The possibility of improving an already adequate diet, with resulting enhancement of nutritional well-being and increase in length of life, has been shown in previous papers (Sherman and Campbell, '24, '28, '30, '35 b).

Using rats as experimental animals, in a study of the proportion of protective food needed to make adequate a food supply otherwise composed of cereal grain, table salt, and distilled water, we found that 1 part of dried whole milk to 5 parts of ground whole wheat (our diet A) was adequate; but that a mixture of one-third whole milk powder with two-thirds ground whole wheat (our diet B) gave better results as judged by observations upon growth, adult vitality, and length of life.

Striking evidence that diet A is adequate, in the full sense in which the word is understood in nutritional research and in human medicine, is afforded by the fact that it supports normal growth and health, reproduction and length of life, generation after generation. (Families are still thriving in the fortieth generation on this diet at the time of writing.) Diet B differed from diet A in the sole experimental variable that it contained a larger proportion of milk. In chemical terms, however, this change involved enrichment in more than

one factor; most markedly in calcium, vitamin A, and the riboflavin factor which in previous papers from this laboratory has been called vitamin G. The new experiments here described were designed to ascertain the factors or fractions of milk which were actually concerned in the improvements of nutritional well-being and length of life which had resulted from the increased proportion of milk in the diet. Careful consideration of the existing state of knowledge led us to plan these experiments partly in terms of the element calcium and partly in terms of the natural fat-soluble and water-soluble fractions of milk. For it was deemed wiser to supply the vitamins A and G (riboflavin) accompanied by their natural concomitants than in purified forms which, in addition to the question of strict chemical purity, would involve (from the viewpoint of this particular investigation) an assumption that no other known or unknown substance could have been a significant factor in the nutritional difference between diets A and B.

EXPERIMENTAL

Diets. Five diets were used, the basis of each being ground whole wheat and dried whole milk: 1) diet A, known in the laboratory as diet 16; 2) to this was added sufficient calcium carbonate to give the same calcium content as in diet B (diet 162); 3) or sufficient butterfat to give the same content of milk fat as in diet B (diet 165); 4) or both calcium carbonate and butterfat in same amounts as in diets 162 and 165 (diet 166); 5) or skim milk powder replacing one-fifth of the wheat, thus enriching the diet in milk proteins, calcium, and riboflavin or vitamin G, and possibly other factors not yet fully known (diet 167). The composition of the diets is given in table 1.

Experimental procedure. Parallel lots of rats, each lot consisting of two males and three females, were made up when the rats were 4 weeks of age, the members in each lot being matched as to weight, sex, and heredity with those in the other lots formed at the same time. All rats used were from families that had been on diet A (diet 16) for twenty generations or more. In this fivefold comparison it was not possible

to place lots of litter mate parallels on every diet each time; but, whenever possible, three such lots were started in parallel, and these were systematically distributed among the diets in rotation so that there were the same number of lots on each diet and the same final parallelism between all diets.

For each of the diets here compared there were twelve fresh starts with lots in direct litter mate comparison with parallel starts on others of the diets, the animals of each such fresh start being known as the 'first generation' on their respective diets except in the case of diet 16 (diet A) which as above explained was the stock diet from which all initial animals were drawn.

TABLE 1

Percentage composition of diets

	DIET 16	DIET 162	DIET 165	DIET 166	DIET 167
Ground whole wheat	81.97	81.66	77.44	77.14	65.79
Whole milk powder	16.39	16.33	16.41	16.35	16.45
Calcium carbonate	......	0.39	......	0.38	......
Butterfat	......	......	4.59	4.58	......
Skim milk powder	......	......	......	......	16.45
Sodium chloride	1.64	1.63	1.56	1.55	1.31
Calories per gram	3.73	3.71	3.98	3.97	3.75
Protein	13.1	13.0	12.6	12.5	16.8
Calcium	0.195	0.337	0.184	0.336	0.367

These initial or first generation animals constituted about one-third of the total numbers included in this comparison, the other two-thirds consisting of their offspring, representatives of which were continued on their parents' diet and were grouped when 4 weeks of age into breeding lots of two males and three females each. Guided by our previous experience as to the number of generations which may be required to show the full effects of relatively small differences of diet, we included in the present comparison representatives of the second, third and fourth generations on diets 162, 165, 166 and 167. These were selected 1) at different seasons of the year, that there might be no unbalanced seasonal influence, 2) so that advanced generations on each of the diets would

be about parallel, and 3) from litters of average weight for the diet.

All experimental animals, either in the first or subsequent generations, were kept until death occurred from natural

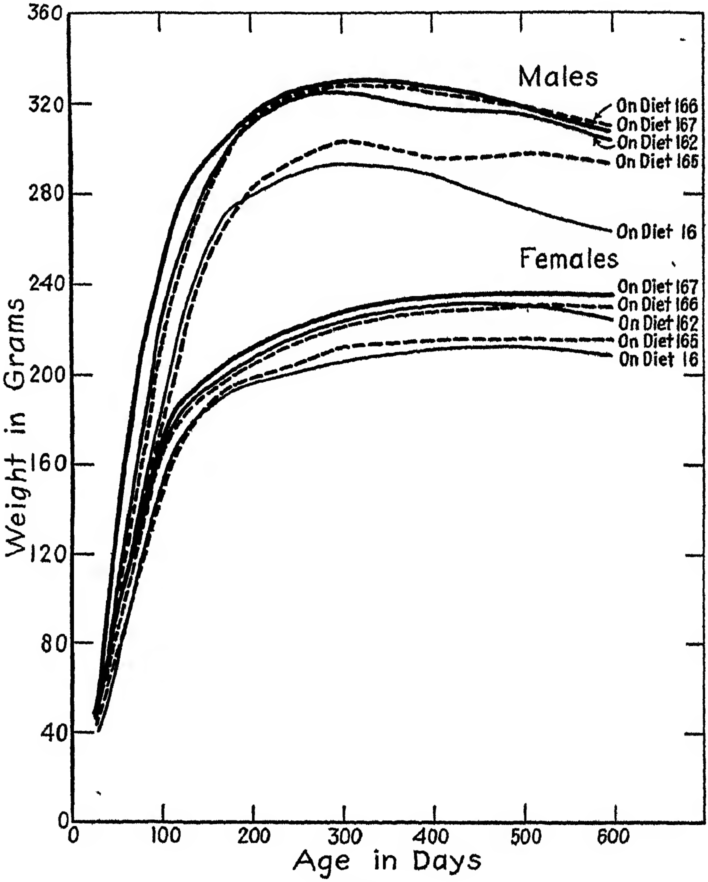

Figure 1

causes, except in some cases where larger numbers were kept for early growth records. The numbers on each diet were from 66 to 77 males, 101 to 111 females, the exact number on each diet being given in table 2, together with the quantitative results of the experiments.

TABLE 2

Comparison of data of experimental animals

DESIGNATION OF DIET	DIET 16	DIET 162	DIET 165	DIET 166	DIET 167
NATURE OF ENRICHMENT	Our basal diet A (no enrichment)	Calcium (as carbonate)	Butterfat (vitamin A)	Calcium and butterfat	Riboflavin, Ca, and protein[1]
Growth from twenty-eighth to fifty-sixth day of age					
Males					
(Number of cases)	(73)	(83)	(66)	(80)	(77)
Gain in grams ± P.E.	56.3 ± 1.05	71.0 ± 0.87	51.0 ± 1.00	66.1 ± 0.86	96.4 ± 1.23
Coefficient of variation	23.9	16.7	24.0	17.4	16.7
Females					
(Number of cases)	(107)	(116)	(102)	(114)	(112)
Gain in grams ± P.E.	47.0 ± 0.69	60.2 ± 0.63	45.2 ± 0.64	57.9 ± 0.52	73.5 ± 0.61
Coefficient of variation	22.8	16.9	21.4	14.4	13.1
Gain per 1000 Calories in lots of two males and three females					
(Number of cases)	(35)	(38)	(36)	(36)	(37)
Grams ± P.E.	64.7 ± 0.94	71.7 ± 0.90	57.8 ± 0.72	66.1 ± 0.81	89.8 ± 1.02
Coefficient of variation	12.9	11.6	11.2	11.1	10.4
Gain per gram of protein in lots of two males and three females					
(Number of cases)	(35)	(38)	(36)	(36)	(37)
Grams ± P.E.	1.83 ± 0.027	2.04 ± 0.026	1.83 ± 0.023	2.02 ± 0.024	2.00 ± 0.023
Coefficient of variation	12.9	11.7	11.2	10.5	10.4
Age at birth of first young					
(Number of cases)	(90)	(98)	(96)	(104)	100
Days ± P.E.	132 ± 2.4	111 ± 1.6	134 ± 2.4	107 ± 1.3	110 ± 2.1
Coefficient of variation	26.1	21.4	26.1	18.3	28.3
Duration of capacity to reproduce					
(Number of cases)	(101)	(102)	(102)	(111)	(109)
Days ± P.E.	213 ± 9.4	279 ± 10.2	286 ± 10.1	241 ± 9.6	234 ± 10.0
Coefficient of variation	66.9	55.3	53.4	63.1	67.0
Young born					
(Number of cases)[2]	(101)	(102)	(102)	(111)	(109)
Young per female ± P.E.	24.1 ± 1.13	32.6 ± 1.34	34.2 ± 1.33	27.7 ± 1.27	27.6 ± 1.26
Coefficient of variation	70.6	62.1	58.9	72.7	71.3
Young reared					
(Number of cases)[2]	(101)	(102)	(102)	(111)	(109)
Young per female ± P.E.	12.7 ± 0.74	20.4 ± 1.12	19.0 ± 0.96	15.3 ± 0.89	16.1 ± 0.94
Coefficient of variation	88.1	83.3	76.4	92.6	91.0
Success in rearing young					
(Number of cases)[3]	(90)	(98)	(96)	(104)	(100)
Per cent young reared	46.0 ± 2.1	56.6 ± 2.0	54.2 ± 1.9	50.8 ± 2.1	53.1 ± 1.9
Coefficient of variation	66.2	52.4	52.6	63.1	54.9
Average weight of young at 28 days					
(Number of cases)	(1286)	(2081)	(1939)	(1688)	(1761)
Grams ± P.E.	38.9 ± 0.08	42.4 ± 0.06	38.9 ± 0.07	40.4 ± 0.08	44.6 ± 0.11
Coefficient of variation	10.9	10.4	11.5	11.9	10.5
Length of life					
Females					
(Number of cases)	(101)	(101)	(102)	(111)	(109)
Days ± P.E.	723 ± 12	746 ± 13	818 ± 11	739 ± 14	754 ± 13
Coefficient of variation	25.3	26.8	20.8	30.0	26.8
Males					
(Number of cases)	(70)	(69)	(66)	(76)	(77)
Days ± P.E.	658 ± 12	703 ± 11	667 ± 14	689 ± 11	681 ± 10
Coefficient of variation	22.8	19.4	26.0	21.0	20.1

[1] As skim milk powder.
[2] Number of females in breeding lots.
[3] Number of females bearing young.

The findings from these experiments have been grouped in sequence according as they afford measures of 1) rate and efficiency of growth and development, 2) adult vitality, and 3) length of life.

DISCUSSION

Rate and efficiency of growth and development. From figure 1 and table 2 it will be seen that enrichment of the basal diet 16 in vitamin A (by adding butterfat) did not increase the rate of growth as measured by gain in weight during the fifth to eighth weeks (inclusive) of the rat's age, or the efficiency of growth as measured in grams of gain per 1000 Calories or per gram of protein consumed, or the rate of development as indicated by the average age of females at birth of their first young when the sexes grew up together. On the other hand, enrichment of the original diet in calcium (with or without the added butterfat) did increase the rate and efficiency of growth and expedited development as reflected in earlier reproduction. When the diet was enriched in riboflavin (and in lesser degree in protein) as well as in calcium, there was a further well-marked increase in the rate of growth with sustained efficiency calculated per gram of protein consumed, and increased efficiency when calculated in terms of gain in weight per 1000 Calories of food intake; but there was no further increase in rate of development over that observed when the diet was enriched in calcium alone.

It should be noted that diet 165, the result of enriching diet 16 by addition of butterfat only, while increasing the intake of vitamin A resulted also, because of its increased fat content, in slight diminution of intakes of protein, mineral elements, and water-soluble vitamins. It is also worthy of note that the addition of butterfat to diet 16, while not increasing the rate of early growth did, result in slightly larger adult size as may be seen from figure 1. Also the final adult weights on the diet enriched in both calcium and butterfat (diet 166) were very slightly above those on the diet enriched in calcium alone (diet 162). On diet 167, which had essentially the same

calcium content as diet 162 but was about one-fourth richer in protein (with a higher proportion of milk protein) and nearly twice as rich in riboflavin, a much more rapid early growth resulted, and a somewhat larger adult size in both sexes. During the early period in which growth was distinctly more rapid on diet 167 there was a somewhat greater food consumption per gram of body weight on this than on the other diets; but after the early period the food consumed per unit of body weight at any given age was practically the same on all five of these diets. The detailed records of food consumption are therefore omitted to save space.

Adult vitality and length of life. Throughout their lives these experimental animals were individually examined and weighed at least weekly. Records of systematic observations of appearance, alertness, condition of skin and coat, and 'feel' of muscular tone have thus been available to aid the interpretation of the numerical data.

Throughout youth and early adult life the appearance of the animals on diet 167 as judged by the condition of the coats, and firmness of body was superior to all others. Animals on diet 165 had a softer hair, and they, as well as those on diets 162 and 166, maintained their youthful appearance longer than those on diet 16.

It was also noticeable that the animals on diet 167 (richer in riboflavin and also of somewhat higher protein content) did not maintain their superiority of physical appearance in their old age. Senility set in at about the same age, and appeared to progress somewhat more rapidly with them than with the animals on diets enriched only with calcium and/or butterfat.

The numerical data used as criteria of adult vitality are 1) the time of maturity of the females as shown by their age at the birth of their first young, 2) their ability to bear and rear young, and 3) the weight of the young at 28 days of age. The outstanding features of these data are summarized in table 2. These later effects of the different enrichments of diet did not in all respects parallel those observed in early

growth. Both calcium and skim milk additions (diets 162, 166 and 167) led to significantly earlier maturity and to higher body weights of offspring; while the females receiving additional calcium or butterfat (diets 162 and 165) showed greater duration of breeding period, larger numbers of young born and reared, and larger percentage of young reared.

While on diet 16 nearly 11% of the females were sterile and 20% of those bearing young failed to rear any, these percentages were reduced on all of the other diets. In other words, the proportion of families enabled to leave descendants was increased when the basal diet was enriched in its calcium content, its butterfat content, or in its content of skim milk solids which was here chiefly significant as markedly increasing the riboflavin as well as the calcium intake, while the protein intake was simultaneously increased in much smaller proportion.

From the data of table 2 it is apparent that, as in the case of the other criteria of nutritional well-being, the increased length of life previously obtained by doubling the amount of milk in the diet (comparisons of diets A and B) was not due to any single factor in the milk, but to a combination of factors. In every case of dietary enrichment here studied, the life span has been somewhat increased; but it is not apparent why males appear to benefit more by the enrichment of the diet in its calcium content and females by the addition of butterfat. A possible explanation of the latter fact may be that the females on diet 165 were on the average about 20 days older when their first young were born, and while they reared as many as were reared on the other diets, the average weight of the young was less, so that the females on this diet might be regarded as having encountered less drain upon their vitality during the reproductive period.

If the increase in length of life on each enriched diet, over the average for the same sex on diet 16, were to be interpreted as if it stood alone, some would and others would not appear to be statistically significant; but, as already explained, each of these differences is a part of the difference hitherto

measured between the effects of our diets A and B upon nutritional well-being and resulting health and length of life. Thus it now becomes apparent that the enrichments in calcium, in vitamin A, and in riboflavin (with its accompanying minor increase in protein) have all contributed to the far-reaching improvement in nutritional well-being which resulted from the better quantitative adjustment in the proportions of natural foods in an already adequate diet.

McCay, Crowell and Maynard ('35) have recorded an experiment in which the slowing of growth apparently increased the average length of life, while in both earlier and later experiments with our diets A and B (Sherman and Campbell, '24, '35 a) we have found the latter to result both in more rapid growth and in longer life. The findings of the two laboratories may be entirely consistent. The starting point of the Cornell experiments was a diet extremely rich in protein and vitamins, whereas ours was an inexpensive dietary composed mainly of wheat and with only slightly above minimal adequate proportions of calcium, of protein and of vitamins A and G (riboflavin). Obviously there is no inconsistency in the view that limitation of intake of a very rich dietary such as theirs, and the enrichment of a dietary such as our diet A, may both be advantageous to the nutritional well-being of the life cycle. There remains a large area of investigation as to what types of diet are advantageous, respectively, to growth, to longevity, and to both. All the experiments in the present paper deal with differences of the order of magnitude of those existing between our diets A and B. Table 2 permits the comparison, for males and females separately, of the data on each of four different enrichments with the data on the basal diet 16 (diet A). Where early growth was slowed by the addition of butterfat to the basal diet, both sexes lived longer; but also when early growth was more rapid on a diet enriched in calcium, in calcium and butter, or in calcium, riboflavin and protein, both sexes here also lived longer on each of the three enriched diets which had expedited their growth. It is consistent both with our

other findings and with those of McCay, Crowell and Maynard that of the diets here studied the one which was richest in protein (and riboflavin) and which induced the most rapid growth in both sexes gave intermediate lengths of life both with females and with males. As pointed out in a previous paper (Sherman and Campbell, '35 b) it may be that the number of young produced and suckled by the females should be considered in connection with their longevity records. We hope to throw further light upon these problems through experiments now in progress.

SUMMARY AND CONCLUSIONS

The complete life cycles were studied in from 66 to 77 males and from 101 to 111 females on each of the five diets here compared. Several objective criteria of nutritional well-being as reflected in growth and development, adult vitality, and length of life, were measured quantitatively and the data submitted to critical statistical examination.

Several chemical factors are thus found to participate in the enhancement of nutritional well-being and increase in the length of life, which resulted from the improvement of an already adequate diet by increasing the proportion of milk in the food supply. The enrichments here studied were in each case of the same degree as the difference between our diets A and B.

Increased intake of calcium, either alone or with butterfat, resulted in definitely more rapid growth; while enrichment with butterfat alone seemed to result in slower growth but larger adult size than on the original diet A (diet 16).

The enrichment of the basal diet in butterfat alone had no measurable effect upon the average age at which the females bore their first young, while each of the other dietary enrichments here studied led to an earlier maturity in this respect.

Butterfat and calcium, either alone or together, resulted in longer life and an extension of the period of adult vitality as measured by the length of time during which the females were capable of bearing young. The addition of butterfat

alone did not increase the size of the young (measured by weight at the age of 28 days, taken as a conventional 'end of infancy' in this rat colony); whereas this was increased by addition of calcium either alone or with butterfat.

With both males and females, addition of butterfat alone was followed by slower growth and longer life; while in the other six of the eight comparisons, i.e., with both males and females on enrichment of diet 1) in calcium, 2) in calcium and butterfat, and 3) in calcium, riboflavin and protein, there was increase both in the rate of growth and in the length of life.

When one-fifth of the wheat of the basal diet was replaced by an equal weight of skim milk powder, i.e., when along with the increase of calcium there was a corresponding increase of riboflavin and a relatively minor increase of protein, growth was significantly more rapid than on any of the other diets; development was expedited and the life cycle extended in essentially the same degree as by enrichment in calcium alone; and larger young resulted than upon any of the other diets studied.

Thus it appears that the three factors which were increased in greatest ratio when the already adequate diet A was modified to constitute the nutritionally better diet B, i.e., calcium, vitamin A, and riboflavin, have all contributed to the improvement in nutritional well-being and resultant health and longevity.

Further studies of the relation between rapidity of growth and length of life as influenced by different modifications of diet are in progress.

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RECOVERY OF CAROTENE AND VITAMIN A FROM BUTTER WHEN COWS WERE FED UNLIMITED QUANTITIES OF GREEN RYE[1]

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The following authors have established the facts that both the carotene and vitamin A contents of butter vary widely, and that they are largely dependent on the carotene of the feeds consumed by the cow (Baumann and Steenbock, '33; Fraps and Treichler, '32; Krauss, '30; Loy, Hilton, Wilbur and Hauge, '37; Palmer and Eckles, '14; Shrewsbury and Kraybill, '33).

Moore ('32) showed that the cow is capable of converting the carotene in her ration into vitamin A in the butter fat she produces. Early investigations (Fraps and Treichler, '32; Moore, '32) have shown that only a small fraction of the total carotene ingested appears in the milk either as carotene or vitamin A.

Meigs and Converse ('33) concluded that a ration of grain and hay or grain, hay and silage was likely to be deficient in vitamin A for liberally milking cows unless it contained large quantities of good quality legume hay. After studying various rations as sources of vitamin A for dairy cows, Fraps, Copeland and Treichler ('34) concluded that apparently pasture grasses were needed to maintain the production of butter fat high in vitamin A. Krauss ('30) reported that cows doubled the vitamin A value of their butter fat when turned on pasture.

[1] Contribution no. 110, Department of Dairy Husbandry; no. 220, Department of Chemistry; and no. 64, Department of Home Economics.

Hilton, Hauge and Wilbur ('33) found that butter with high vitamin A value could be produced in winter provided good quality alfalfa or soy bean hay was fed.

Moore ('32) reported that the carotene and vitamin A content of butter never exceeds that of normal summer butter regardless of the amount consumed in the feeds. Treichler, Grimes and Fraps ('35) fed cows on a carotene-poor, vitamin A deficient diet for 60 days and then placed them on pasture for 14 days. The vitamin A content of the butter was increased from 12 units per gram to a maximum of 50 units, while the carotene increased from 0.53 to 8.03 γ per gram of butter. The amount of carotene ingested daily while the cows were on pasture was not known.

Baumann, Steenbock, Beeson and Rupel ('34) made several quantitative studies of the carotene consumed in the feeds by different breeds of dairy cows and the carotene and vitamin A recovered in the butter fat. On a winter ration the average daily intake of carotene per cow was equivalent to 240,000 international units of vitamin A, but only 8244 units were secreted in the milk. When the hay and silage of the winter ration were replaced with freshly cut alfalfa hay to simulate pasture conditions, an average of 1,040,000 units were ingested daily per cow and only 14,000 units were recovered in the butter fat. Of the carotene ingested on the winter ration an average of 1.12% appeared in the butter fat and 0.4% on the summer ration. The vitamin A in the butter fat from the two rations represented only 1.6 and 0.7% respectively of the carotene in the rations. Most of the cows in the Wisconsin experiment were consuming about 50 pounds of green material, with an average maximum of 65 pounds, whereas it is known (Woodward, '36) that cows will consume nearly three times that amount of fresh green grass.

Although several investigators have reported the carotene and vitamin A content of butter when cows are on pasture, the above experiment is the only one known to the writers in which the daily intake of carotene was known.

In view of these facts it seemed desirable to extend these studies to include cows which were consuming fresh green feed in larger quantities and the intake of carotene known, in order to measure the recovery of carotene and vitamin A in butter under pasturage conditions.

EXPERIMENTAL

The three cows used, two Ayrshires and one Holstein, were typical pure breds and averaged about a pound of butter fat daily in production. For several months previous the cows had received the regular herd grain mixture, sorghum silage and alfalfa hay. During the experiment the cows were individually stall fed green rye only. Each cow was offered in two feeds daily all the rye she would consume. A sufficient supply for 1 day's feeding was cut early each morning. The rye had been pastured previously and was approximately 15 inches high. It was not as green and succulent as previously and about one-fourth of the plants had headed. The cows were fed green rye 16 days, the last 3 days being used as the experimental period.

TABLE 1
Description and production of cows used

EAR TAG NUMBER OF COW	BREED	BODY WEIGHT	GREEN RYE CONSUMED DAILY	WATER IN RYE	DRY MATTER CONSUMED DAILY	MILK PRODUCED DAILY	FAT IN MILK	FAT PRODUCED DAILY
		pounds	*pounds*	%	*pounds*	*pounds*	%	*pounds*
239	Ayrshire	955	136.5	82.3	23.9	37.2	3.8	1.4136
240	Ayrshire	1120	143.1	82.3	25.0	22.5	4.0	0.9000
118	Holstein	1196	144.2	82.3	25.2	21.2	4.0	0.8480
Average			141.3	82.3	24.7	27.0	3.9	1.0539

A representative sample of the rye was prepared for analysis by cutting as finely as possible with shears immediately after collection. Carotene analysis was made according to previously described technic (Guilbert, '34; Peterson, Hughes and Freeman, '37). Butter samples for analysis were obtained during the last 3 days of the rye feeding period by saving all the milk produced daily by each cow. The milk from each

cow was separated in a small cream separator and the complete 3-day collection of cream churned by hand. The carotene content of each of the three butter samples was determined spectophotometrically on the petroleum phasic fractions of aliquots which had been hydrolyzed and separated in the usual manner (Guilbert, '34). Vitamin A was determined on the non-saponifiable residues of each of the three butter samples (Report of the sub-committee on determination of unsaponifiable matter in oils and fats, '33) and the concentration determined from the intensities of the absorption band at 328 μ, as obtained from plates exposed in a Bausch and Lomb quartz

TABLE 2

Carotene content of rye, and the carotene and vitamin A content of butter

EAR TAG NUMBER OF COW	CAROTENE CONTENT PER POUND			VITAMIN A PER GRAM (EXCLUSIVE OF CAROTENE)		
	Fat in butter	Butter	Butter fat	Butter	Butter fat	
	%	*mg.*	*mg.*	*gamma*	*gamma*	*I.U.*[1]
239	82.7	2.52	3.05	10.4	12.6	19.7
240	86.5	2.27	2.62	10.1	11.8	18.4
118	84.1	2.35	2.79	9.7	11.5	17.9
Average	84.4	2.38	2.82	10.1	12.0	18.7

[1] One gamma of vitamin A = 1.56 I.U. (Morgan, R. S., J. Edisburg and R. A. Morton. 1935. Biochem. J., vol. 29, p. 1645).

NOTE: Green rye contained 25.1 mg. carotene per pound as fed, or 142.0 mg. on a dry basis.

spectrograph equipped with a Hilger rotating disc and quartz biprism. Though the ultraviolet absorption at 328 μ has now been generally accepted to be a measure of the content of vitamin A (League of Nations Commission, '34), we are aware that the method has its limitations. It is questionable, for example, whether all substances other than vitamin A which absorb at 328 μ can readily be eliminated without any loss of vitamin A. Furthermore, Pritchard and Wilkinson ('37), studying the discrepancies between biological and physico-chemical methods of vitamin A assay, have reported the existence of fractions which do not correspond with the physical and chemical criteria usually accepted for vitamin A, but are biologically very active.

DISCUSSION

The carotene ingested daily averaged 3.507 gm., equivalent to nearly 6,000,000 international units of vitamin A. This emphasizes the enormous intake of carotene when cows are on pasture. Under such conditions it is not surprising that cows which have been on low carotene diets for some time have been found to re-establish the carotene and vitamin A content of their butter to a point equal to summer butter in from 2 to 3 weeks when turned onto pasture (Treichler, Grimes and Fraps, '35). It is of interest to note that the three cows studied consumed practically the same amount of dry matter and as a result had approximately the same intake of carotene.

The butter produced by the cows had the following carotene content in international units of vitamin A per pound: no. 239—5080, no. 240—4370 and no. 118—4650, averaging 4700 units. Vitamin A content of the butter in international units for the respective cows was 8944, 8354 and 8127, averaging 8490. Although these values are lower than has been reported by some authors (Sherman, '37), they are quite similar to results obtained for butter from the same breeds at the Wisconsin station (Baumann, Steenbock, Beeson and Rupel, '34), when cows were stall fed 50 pounds of green feed. They are likewise in reasonable harmony with values obtained on butter produced from pasturage at the Texas station (Treichler, Grimes and Fraps, '35), especially considering the fact that in the latter work the total vitamin A value was determined by biological analysis and the vitamin per se obtained by subtracting the value of the carotene from the total.

Thus by comparison it may be concluded that the feeding of carotene at the rate of approximately 1,000,000 international units daily resulted in butter typical of grass produced butter; but when the daily intake of carotene was increased to approximately 6,000,000 units, which is probably more typical of pasturage conditions, the carotene and vitamin A content of the butter remained relatively unchanged. This is in harmony with Moore's ('32) statement that the carotene and vitamin A

content of butter cannot be increased beyond the normal of typical summer butter.

Great waste in the utilization of carotene must, therefore, be expected under pasturage conditions. In this experiment, where an average of 5,846,000 international units were fed as carotene, an average of only 0.086% of carotene was recovered daily in the butter, while 0.154% was recovered as vitamin A, or a total of 0.24% in terms of international units. When about one-sixth as much carotene was fed, Baumann et al. ('34) recovered in the butter an average of 1.12% as carotene and 0.4% as vitamin A, making a total of 1.52%, or approximately six times the percentage recovered in the case where six times as much was fed. On winter rations

TABLE 3

Daily carotene intake of cows and daily recovery of carotene and vitamin A in butter (in international units)

EAR TAG NUMBER OF COW	DAILY INTAKE OF CAROTENE	DAILY RECOVERY			PER CENT VITAMIN A POTENCY DUE TO CAROTENE	PER CENT RECOVERED		
		Carotene	Vitamin A	Total		Carotene	Vitamin A	Total
239	5,656,000	7186	12,643	19,829	36.2	0.127	0.224	0.351
240	5,917,000	3930	7,518	11,448	34.3	0.066	0.127	0.193
118	5,964,000	3943	6,892	10,835	36.4	0.066	0.116	0.182
Average	5,846,000	5020	9,014	14,034	35.5	0.086	0.154	0.240

(240,000 units daily) they recovered in the butter 1.6% as carotene and 0.7% as vitamin A. Not only is a relatively small proportion of the carotene ingested recovered in the butter fat but apparently the greater the amount consumed the smaller the percentage recovered, at least, when generous amounts are provided.

It is interesting to note the close agreement of the results obtained on the three cows in regard to "per cent of vitamin A value in the butter due to carotene" (table 3). The average figure of 35.5% is in close accord with an average of about 32% computed for the same breeds from the results reported on their summer ration by Baumann, Steenbock, Beeson and Rupel ('34). In fact, even though the vitamin A was determined by a different method, i.e., biological assay, the report

of Treichler, Grimes and Fraps ('35) is in harmony with these results for cows on pasture. They reported 23, 30, 33 and 41 for four Jersey cows, a breed which is known to produce butter relatively higher in carotene in proportion to vitamin A than are Holsteins and Ayrshires.

It would seem, therefore, that when excessive amounts of carotene are fed daily there is tendency for the ratio of carotene to vitamin A per se in the butter to become rather constant at about 1 part of carotene to 2 parts of vitamin A, at least with Holsteins and Ayrshires (high vitamin A and low carotene breeds).

The fact that the three cows varied considerably in daily production might indicate that the ratio of carotene to vitamin A in the butter is not influenced by plane of production. Examination of the data reported by Baumann et al. ('34), and by Treichler et al. ('35) indicates that this constant ratio probably would not prevail at lower levels of carotene intake. In fact, the latter work might indicate that as the carotene in the ration is reduced, the percentage of the total vitamin A value of the butter as carotene is reduced.

SUMMARY

To simulate pasturage conditions, three cows, two Ayrshires and one Holstein, were fed all the fresh green rye they would consume for 16 days. During the last 3 days the carotene ingested daily, and the carotene and vitamin A output in the milk were computed. Daily intake of carotene averaged 3.507 gm., equivalent to nearly 6,000,000 international units of vitamin A. The butter produced had an average carotene content per pound of 4700 international units, and 8490 units as vitamin A, making a total vitamin A value of 13,190 international units. Of the carotene ingested daily only an average of 0.086% was recovered daily as carotene in the butter and only an average of 0.154% as vitamin A, or a total of 0.24% in terms of international units. The percentage of the total vitamin A value in the butter due to carotene was quite uniform for the three cows, averaging 35.5%. The data seem to

indicate that when excessive amounts of carotene are fed, the ratio of carotene to vitamin A in the butter becomes rather constant at about 1 to 2, at least with Holsteins and Ayrshires (high vitamin A and low carotene breeds).

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LOSSES OF VITAMIN C DURING THE COOKING OF SWISS CHARD[1]

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TWO FIGURES

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Swiss chard was chosen for study because it is an easily grown hardy vegetable with a long growing season, also because it is rich in minerals and, presumably, also in vitamin C as are spinach and other greens. The only study on the vitamin C of chard found in the literature was that carried out by Wasson ('31). This experiment was on raw chard only.

Wasson ('31) reports that 2 gm. daily of the green leafy portion (with the white rib removed) of fresh raw Swiss chard was not sufficient to prevent scurvy in guinea pigs; that even 4 gm. prevented scurvy in only one case out of six although all the animals gained in weight. She also found that 4 gm. of the white rib was not protective. Recalculated on the basis that 0.5 mg. ascorbic acid per day is required to protect a guinea pig from scurvy (Bessey and King, '33), this means that the green leafy chard contained less than 0.125 mg. of ascorbic acid per gram and the white rib considerably less than this amount. Wasson ('31) concludes that "the vitamin C potency of Swiss chard is very low." She did not name the variety of chard used or the kind of soil on which it was

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[2] Buhl Foundation Research Fellow. Publication no. 347 from the chemistry department, University of Pittsburgh.

grown. The chard was freshly cut, but Wasson reports that the animals on the green leafy portion were slow about eating it and some of them would have part of the chard left in their cages after 5 or 6 hours. This would probably decrease the amount of vitamin C present in the chard by the time it was consumed.

The purpose of this experiment was to determine not only the vitamin C content of the raw chard, but also that of the cooked chard and the cooking water at the 'done' stage and at various intermediate stages. The amounts of ascorbic acid reported are those obtained after reduction with hydrogen sulphide inasmuch as this represents the biologically active vitamin C present. The aim in cooking was to make the cooked vegetable look attractive and taste well, with the loss of nutrients a secondary consideration.

EXPERIMENTAL

Two varieties of Swiss chard, Lucullus and Fordhook, were grown on upland Ontario clay loam soil at Geneva, New York, and were cut during July. The Lucullus is dark green and is the market preference at present due to the fact that it does not wilt as readily as the lighter colored chard. Both varieties have large white midribs. Vitamin C determinations were made of the leaf with the midrib left intact, with the midrib removed, and of the stems alone. Analysis was also made of leaves which were chopped and those which were cut with scissors. The chard was gathered, washed, the clinging moisture removed by means of electric fans, the stems removed, the uncut leaves cooked and analyzed in as short a time as possible.

In cooking, a 3-quart enamel pan with an inside diameter of 7 inches was used. The pan and gas burner were placed on a balance so that the weight of the pan and its contents was obtainable at any stage. A manometer was connected between the gas supply and stove so that it was possible to regulate the amount of evaporation of the cooking water making it practically the same in successive cookings. Three hundred

and fifty grams of chard (four servings) were used with 1200 cc. of water and $1\frac{1}{2}$ teaspoons of salt. The chard was dropped into the rapidly boiling water and the time counted from the beginning of cooking, as the water did not stop boiling. The tap water was boiled several minutes before the chard was added. An arbitrary stage of 'doneness' was determined in advance by several judges, testing with a fork and 'biting.' The boiling time for both varieties was 10 minutes. Due to the fact that it was impossible to secure samples of whole leaves (vitamin C was found to be unevenly distributed in the green leafy part and the white portion of the midrib) during the cooking and also to standardize the draining of the samples it was found necessary to have separate cookings for each of the intervals analyzed, namely, 2, 4, 6, 8, 10 ('done'), and 14 ('overdone') minutes. At the end of each of these cooking periods the chard was drained for 1 minute, samples of whole leaves removed and the remainder weighed immediately. Samples of the cooking water were also removed and the remainder weighed immediately. The weights of the total chard and the total cooking water were thus easily obtained.

After trying 8% acetic, 8% trichloracetic, 4% lactic, 1 N and 2 N sulphuric acids, all containing 2% metaphosphoric acid, it was decided to use 8% acetic acid for extracting as it gave as clear a solution as any of the acids and also resulted in a color which gave as little trouble as any acid in duplicating the titration end point.

The method of sampling was the same as that reported by Fenton, Tressler and King ('36). The method of extraction, of standardization of the dye, and titration were essentially those of Bessey and King ('33) but modified by using for the extraction an acid solution containing 2% metaphosphoric acid according to Mack and Tressler ('37). The 2,6 dichlorophenolindophenol dye was standardized with pure ascorbic acid. A total of 100 cc. of acid was used, 30 cc. being used in grinding the chard with sand in a mortar; the remainder was used in three rinsings. The first mixture was centrifuged and the liquid decanted into a 100-cc. volumetric flask. The rins-

ings were added, the mixture centrifuged and the liquid decanted between each addition of acid. Aliquots were titrated with the dichlorophenolindophenol dye immediately.

Since it was found that during extraction with the acetic metaphosphoric acid mixture there was considerable oxidation of the titratable ascorbic acid to the dehydro form, aliquots were taken and the reversibly oxidized ascorbic acid was reduced immediately according to the general method of Tillmans, Hirsch and Jackisch ('32) with the following modification: hydrogen sulphide was bubbled slowly through the sample for 10 minutes, the container stoppered and allowed to stand 20 minutes. The excess of hydrogen sulphide was removed with a rapid stream of carbon dioxide. The samples were then titrated with the dye, providing a measure of the total vitamin C content.

For purposes of comparison, biological assays were made on the raw and 'done' chard. The whole lot of one variety (Lucullus) for animal feeding was gathered the same day. Part of it was frozen directly with no blanching while part of it was cooked and drained, cooled in shallow pans surrounded by ice and salt and then packed, sealed, frozen, and kept in a container with dry ice.

The curative type biological assay was used, as in the earlier investigations of this series (Tressler, Mack and King, '36). Guinea pigs weighing approximately 225 to 250 gm. were placed in individual cages where they received the modified Sherman et al. basal diet ('22) (oats, bran, milk powder, irradiated yeast, salt and butterfat) and spinach until they had reached an average weight of approximately 260 gm. Animals that were subnormal in growth rate or significantly abnormal in any respect were discarded. They were then depleted of their tissue reserves of vitamin C by being kept on the basal diet only for 13 days. Weighed quantities of the test foods were fed daily during the ensuing 21 days, the quantity fed being based upon the dichlorophenolindophenol titration value after reduction with hydrogen sulphide. A feeding level of 0.5 mg. per day permits only partial recovery from mild

scurvy during the 21-day period, hence this level is suitable for optimum sensitivity.

A summary of the data is given in table 1. It is evident that the biological response of the three groups was comparable in both weight change and scurvy score. The record for the raw chard group was not quite as good as for the other two groups, however. This was probably due in part to the rapid rate of decomposition of the dehydro-ascorbic acid into its irreversible product.

TABLE 1

Biological assay of the antiscorbutic value of raw and cooked chard after storage in dry ice

TEST FOOD	WEIGHT [1] OF CHARD FED	VITAMIN LEVEL	NUMBER OF ANIMALS	AVERAGE WEIGHT AT BEGINNING OF DEPLETION	AVERAGE WEIGHT AT BEGINNING OF FEEDING PERIOD	AVERAGE WEIGHT AFTER 21 DAYS	AVERAGE GAIN	AVERAGE SCURVY SCORE
	gm.	*mg. per day*		*gm.*	*gm.*	*gm.*	*gm.*	
Vitamin solution		0.5	8	263	272	284	+ 12	3
Raw chard	1.42	0.5	6	251	291	290	— 1	4
Cooked chard	3.12	0.5	8	253	276	294	+ 18	2
Vitamin solution		1.0	4	268	284	348	+ 64	1
Negative control		0.0	4	260	298	176 [2]	—122	16

[1] Quantity fed based upon values obtained from preliminary and concurrent dichlorophenolindophenol titrations.

[2] Average weight at end of survival period.

The titration values of the raw and the cooked chard used in the biological assays, after storage in dry ice, at the beginning of and during the test period did not show significant variation beyond that inherent in sampling such products. The raw chard contained 0.35 mg. of ascorbic acid per gram, while the cooked chard contained only 0.16 mg. of ascorbic acid per gram. The reduction and titration of the vegetable extracts were always checked with a standard vitamin solution to avoid an error due to residual hydrogen sulphide or to reoxidation of the vitamin. The rate of loss in dehydro-ascorbic acid was about 50% every 3 hours when the frozen raw unblanched chard was allowed to stand exposed at room temperature.

DISCUSSION

The results of titration calculated on the wet and dry basis of the chard per gram and the results of titration of the cooking water per cubic centimeter are presented in table 2. The data in table 2 are presented graphically by figures 1 and 2. The curves so constructed show the milligrams of vitamin C per gram of the raw and cooked chard (on both wet and dry bases) and per cubic centimeter of the cooking water. The data in table 3 show the vitamin C in the total chard and in the total cooking water at various cooking intervals, also the percentage loss of vitamin C from the chard and the percentage gain to the cooking water.

The vitamin C content of the green portion of the leaves with the midrib cut out was found to be about 0.40 mg. per gram, while that of the stems was 0.08 mg. per gram (wet weight). The midrib was left in the leaves (although the stems were cut off) during the experiment as the housewife does not discard the midrib. Furthermore, it was found that upon removing the midrib the cooked leaves contained only two-thirds the amount of vitamin C per gram that they did if the midrib was left intact. This loss from the cut surface is to be expected in view of the great solubility of the vitamin in water. The presence of the midrib meant considerable variation in results, as the proportion of midrib varied in size in different leaves. The size of the leaves varied somewhat also. The extent of drainage proved to be a variable factor. There was less loss of vitamin C from raw chard when it was cut with scissors than when it was chopped with a knife due perhaps to less bruising of the tissues.

The Lucullus variety of chard contained slightly more vitamin C than did the Fordhook variety. This may be partially explained by the fact that the latter contained a larger proportion of midrib.

The average vitamin C content of the raw chard, 0.40 and 0.37 mg. per gram (wet weight) and 3.74 and 3.60 mg. per gram (dry weight), for the Lucullus and the Fordhook varieties, respectively, was greater than that found by Wasson

TABLE 2

Vitamin C losses from Swiss chard (per gram) and gain in the cooking water (per cubic centimeter)[1]

LENGTH OF COOKING PERIOD IN MINUTES[2]	LUCULLUS VARIETY			FORDHOOK VARIETY		
	Ascorbic acid			Ascorbic acid		
	Milligram per gram drained chard		Milligram per cubic centimeter cooking water	Milligram per gram drained chard		Milligram per cubic centimeter cooking water
	Wet weight[3]	Dry weight		Wet weight[4]	Dry weight	
Averages						
0 (raw)	0.43	3.8	0	0.36	3.6	0
2	0.25	2.6	0.04	0.22	2.5	0.02
4	0.21	2.4	0.05	0.19	2.3	0.03
6	0.16	1.9	0.07	0.17	2.0	0.04
8	0.15	1.8	0.08	0.14	1.8	0.05
10 (done)	0.16	1.8	0.09	0.12	1.3	0.06
Averages						
0 (raw)	0.37	3.7	0	0.37	3.6	0
10 (done)	0.16	1.8	0.07	0.14	1.6	0.06
14 (overdone)	0.14	1.2	0.10	0.10	1.0	0.08

[1] There was no interference from sampling in any of the cookings. The amounts of ascorbic acid shown in this table were those found after reduction with hydrogen sulphide.

[2] In all cases the water did not stop boiling when the chard was added.

[3] In terms of the raw vegetable these values were 0.43, 0.28, 0.24, 0.18, 0.17, 0.17, 0.37, 0.18 and 0.11, respectively.

[4] In terms of the raw vegetable these values were 0.36, 0.24, 0.22, 0.19, 0.16, 0.14, 0.37, 0.15 and 0.10, respectively.

TABLE 3

Total vitamin C in the chard and in the cooking water[1]

LENGTH OF COOKING PERIOD IN MINUTES[2]	LUCULLUS VARIETY				FORDHOOK VARIETY			
	Ascorbic acid in milligram		% of original ascorbic acid in		Ascorbic acid in milligram		% of original ascorbic acid in	
	Drained chard	Cooking water	Drained chard	Cooking water	Drained chard	Cooking water	Drained chard	Cooking water
Averages								
0 (raw)	151	0	100	0	125	0	100	0
2	100	37	66	24	85	23	68	18
4	85	58	56	38	77	33	61	26
6	64	72	42	48	66	44	53	35
8	60	77	40	51	58	51	46	41
10 (done)	59	80	39	53	50	56	40	45
Averages								
0 (raw)	134	0	100	0	130	0	100	0
10 (done)	62	66	46	49	55	57	42	44
14 (overdone)	39	83	29	62	32	73	25	56

[1] There was no interference from sampling in any of the cookings. The amounts of ascorbic acid shown in this table were those found after reduction with hydrogen sulphide.

[2] In all cases the water did not stop boiling when the chard was added.

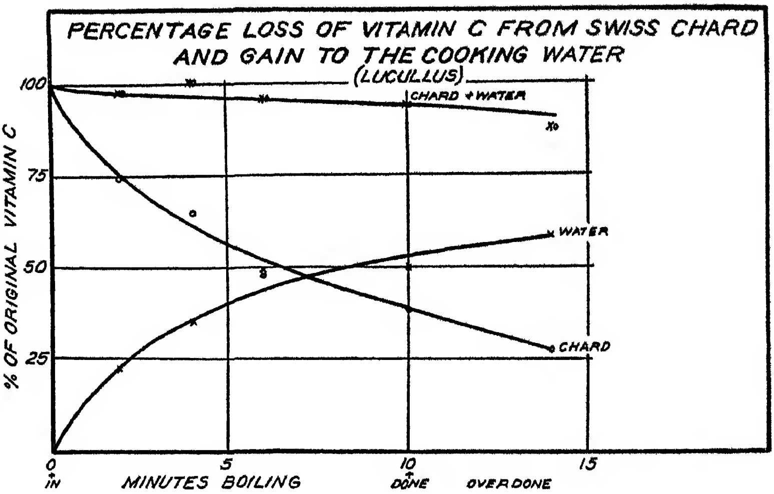

Figure 1

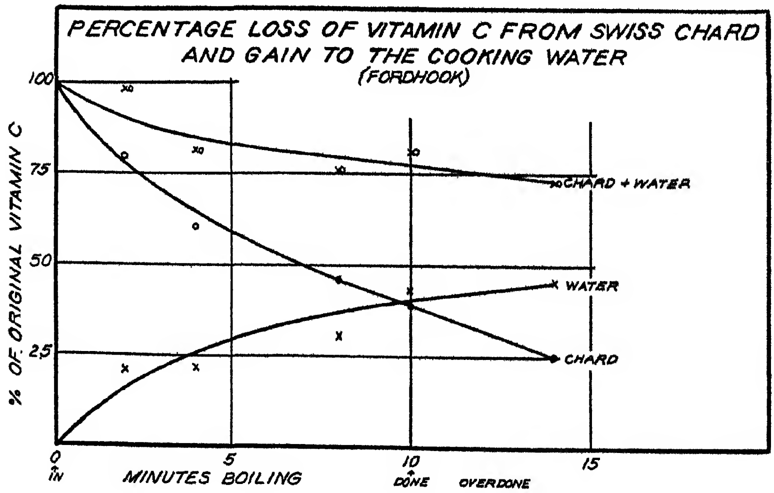

Figure 2

('31) (0.125 mg. per gram wet weight in the green leafy portion, assuming that 0.5 mg. ascorbic acid per day is required to protect a guinea pig from scurvy). In both cases the chard was freshly cut. Since the destruction of the vitamin by oxidation is very rapid in chard, Wasson's low values may be partially explained by the fact that the animals on the green portion of the leaf were always slow about eating it and some of them would have part of the chard left in their cages after 5 or 6 hours.

The fact that the reduced and the reduced plus the reversibly oxidized ascorbic acid were nearly the same after the first 2 minutes of cooking indicates that probably the so-called ascorbic acid oxidizing enzyme was inactivated during this short period. This finding agrees with that of Fenton, Tressler and King ('36) in a study of peas. Kertesz, Dearborn and Mack ('36) report that the ascorbic acid oxidase in other vegetables is inactivated by heating at 100°C. for 1 minute. The rate of loss after the first 2 minutes of cooking was decreased and most of the loss was found in the cooking water.

During a standard cooking period about half of the vitamin C was dissolved in the water. Vinokurov and co-workers ('35), Halliday and Noble ('36), Fenton, Tressler and King ('36) and Gould, Tressler and King ('36) found similarly large amounts of vitamin C lost from other vegetables to the cooking water.

The average destruction of vitamin C in the Lucullus variety at the 'done' stage was 6%, 41% being retained in the chard and 53% in the cooking water. The total destruction in the Fordhook variety at the 'done' stage was 16%, 40% being retained in the chard and 44% in the cooking water.

SUMMARY

1. The leaf portion of fresh raw Swiss chard is a good source of vitamin C (the leaf minus the stem contains about 0.38 mg. per gram).

2. The stem of fresh raw Swiss chard is a relatively poor source of vitamin C (about 0.08 mg. per gram).

3. Cooked chard contains from 0.14 to 0.18 mg. of vitamin C per gram.

4. The two varieties of Swiss chard studied contained about the same amount of vitamin C.

5. About one-half of the original vitamin C passes into the cooking water by the method of cooking used.

6. The so-called ascorbic acid oxidizing enzyme is very active in Swiss chard as indicated by the large amount of dehydro-ascorbic acid found in raw chard after extraction with acetic and metaphosphoric acid. The enzyme is inactivated during the first few minutes of cooking.

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INDEX